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# **Investigating the role of IL-4/IL-13 and their receptors in ulcerative colitis.**

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Thesis submitted to the University of Cape Town in fulfilment of the degree  
Doctor of Philosophy

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## **DECLARATION**

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**ABBREVIATIONS**

aaMph's	-	Alternatively activated macrophages
$\alpha$ -GalCer	-	$\alpha$ -galactosylceramide
Abs	-	Antibodies
Alum	-	Aluminium hydroxide
AOM	-	Azoxymethane
AP	-	Alkaline phosphatase
AP-1	-	Activator protein-1
APC	-	Allophycocyanin
APCs	-	Antigen presenting cells
5-ASA	-	5-Aminosalicylates
$\beta$ 2-ME	-	Beta-Mercaptoethanol
BCA	-	Bicinchoninic Acid Protein Estimation
Bio	-	Biotinylated
BM	-	Bone marrow
CD	-	Crohn's disease
Cpm	-	Counts per minute
Cre	-	Cyclization recombinase
CRP	-	C-reactive protein
CRF2-4	-	Cyto receptor family 2-4
DAI	-	Disease activity index
DCs	-	Dendritic cells
DSS	-	Dextran sulphate sodium
EBI3	-	Epstein-Barr virus-induced gene 3
ELISA	-	Enzyme-linked Immunosorbent Assay
EPO	-	Eosinophil protease
Etoh	-	Ethanol
FACS	-	Fluorescence-activated cell sorter
FCS	-	Foetal calf serum
FITC	-	Fluorescein isothiocyanate
$\gamma\delta$	-	Gamma delta T cells
GM-CSF	-	Granulocyte macrophage-colony stimulating factor
H&E	-	Haematoxylin and eosin
HRP	-	Horseradish peroxidase

IBD	-	Inflammatory bowel disease
IFN- $\gamma$	-	Interferon-gamma
Ig	-	Immunoglobulin
IL	-	Interleukin
IL-4R $\alpha$	-	Interleukin-4 receptor-alpha
IL-12 $\beta$ 2	-	Interleukin-12 receptor-beta2
IL-13R $\alpha$ 1	-	Interleukin-13 receptor-alpha1
IL-13R $\alpha$ 2	-	Interleukin-13 receptor-alpha2
IMDM	-	Iscoves Modified Dulbeccos Medium
iNKT	-	Invariant natural killer cells
I.p	-	Intraperitoneal
IRS	-	Insulin receptor substrate
JAK	-	Janus tyrosine kinases
<i>L. major</i>	-	<i>Leishmania major</i>
LN	-	Lymph Node
mAbs	-	Monoclonal antibodies
MACS	-	Magnetic labelled bead cell separation
Mbtps1	-	Membrane-bound transcription factor peptidase site 1
MHC	-	Major histocompatibility complex
MPO	-	Myeloperoxidase
MyD88	-	Myeloid differentiation primary response gene (88)
<i>N. brasiliensis</i>	-	<i>Nippostrongylus brasiliensis</i>
ND	-	Not detected
NEAA	-	Non-essential amino acids
NFAT	-	Nuclear factor of activated T cells
NK	-	Natural killer
NKT	-	Natural killer T cell
OD	-	Optical density
OTUD3	-	OTU domain containing 3
Ova	-	Ovalbumin
Oxa	-	Oxazolone
PAMPS	-	Pathogen-associated molecular patterns
PAS	-	Periodic acid-Schiff reagent
PBS	-	Phosphate buffered saline

PCR	-	Polymerase chain reaction
PE	-	Phycoerythrin
PLA2G2E	-	Phospholipase A2 group IIE
PMA	-	Phorbol 12-myristate 13-acetate
PNP	-	4-Nitrophenylphosphate
RBC	-	Red blood cell
RNF186	-	Ring finger protein
SCID	-	Severe combined immunodeficiency
SEM	-	Standard error of the mean
<i>S. Mansoni</i>	-	<i>Schistosoma mansoni</i>
STAT	-	Signal transducer and activator of transcription
T-bet	-	T-box expressed in T cells
TCR	-	T cell receptor
Th	-	T helper
TLR	-	Toll-like receptors
TNBS	-	Trinitrobenzene sulphonic acid
UC	-	Ulcerative colitis
WASP	-	Wiskott-Aldrich syndrome protein

# **Abstract**

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## ABSTRACT

Ulcerative colitis (UC) is a heterogeneous inflammatory bowel disease (IBD) associated with chronic inflammation of the gastrointestinal tract. Characterized by genetic and immunological abnormalities, UC has overly aggressive T-cell responses to commensal bacteria eventually leading to disease pathology. UC is distinguished from Crohn's disease, another form of IBD, in that it is driven by a T helper type 2 (Th2) immune response. Oxazolone-induced colitis is a mouse model resembling UC presenting with inflammation limited to the distal colon and mixed neutrophil/lymphocyte infiltration in the superficial layer of the mucosa. The Th2 cytokines interleukin (IL)-4 and IL-13 are associated with the onset of oxazolone colitis and both signal through a common IL-4 receptor-alpha chain (IL-4R $\alpha$ ). Neutralizing these cytokines prevents or ameliorates disease significantly, while neutralizing IL-12 exacerbates disease symptoms. As many aspects of the mechanisms involving Th2 cytokines in colitis remain undefined, the aim of this study was to investigate the role of IL-4 and IL-13 and the receptors through which they signal in oxazolone-induced colitis.

Previous studies have highlighted a role for IL-4 and IL-13 in mediating oxazolone colitis. We show that while IL-13-deficient BALB/c mice were protected from disease onset, IL-4R $\alpha$ -deficient BALB/c mice developed exacerbated disease symptoms. Removing IL-13 production from IL-4R $\alpha$ -deficient mice reversed the susceptible phenotype to protection. These results demonstrate that IL-13 is a major disease-causing factor, able to signal independently of the IL-4R $\alpha$  chain. It is likely that IL-13 signals via the IL-13R $\alpha$ 2 chain, previously believed to be a decoy receptor for IL-13 only, but more recently shown to transduce a signal in cells. Furthermore, the exacerbated phenotype in IL-4R $\alpha$ -deficient mice may suggest that IL-4R $\alpha$ -responsiveness also influences disease. To determine the possible role of IL-4R $\alpha$ -responsive lymphocytes in colitis, we investigated mice lacking this receptor specifically on B cells (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) or T cells (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>). Interestingly, both cell specific mouse strains were protected from disease. Protection correlated with impaired IgE antibody responses in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice and neutralizing IgE significantly protected BALB/c mice from the onset of colitis. Adoptively transferring CD4<sup>+</sup> T helper cells into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice reversed protection back to a susceptible phenotype with increased IL-13 production. Together, these data suggest that IL-4 responsive CD4<sup>+</sup> T cells and IgE-producing B cells are crucial in mediating oxazolone-induced colitis.

Together, our study describes cellular mechanisms underlying oxazolone colitis and shows an important role for IL-4R $\alpha$  signalling in the induction of disease. Similar processes may be involved in human ulcerative colitis and blocking various signalling pathways could equally protect from disease development.

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# **Chapter 1: Introduction**

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### 1.1. Innate and Adaptive Immunity

The mammalian immune system has evolved to allow recognition and elimination of pathogens and foreign material (antigens) while maintaining tolerance towards 'self' components (Davidson 1985; Roitt *et al.* 2001). The innate immune response precedes adaptive immunity following infection or antigen recognition. A number of cell types and serum proteins provide immediate protective responses, following contact with pathogens. The responses generally triggered by pattern recognition provide stimuli that shape the adaptive immune system. Important to the innate response are phagocytic macrophages and neutrophils. These cells bind to micro-organisms, internalize and kill them (Solomon *et al.* 1990). Eosinophils are triggered to release components such as eosinophil protease (EPO), which target parasites too large to be phagocytosed. They also release enzymes that inactivate mast cell products, and therefore decrease inflammatory responses (Solomon *et al.* 1990). Natural Killer (NK) cells are large granular lymphocytes involved in recognizing and killing cells infected by intracellular pathogens. They have been shown to be the predominant source of IFN- $\gamma$  produced early in the immune response (Scharton and Scott 1993), while NK T cells have been identified as major IL-4 producers in innate immunity (Yoshimoto and Paul 1994). Furthermore,  $\gamma\delta$  T cells respond directly to pathogen-associated molecular patterns (PAMPS) independent of major histocompatibility complex (MHC) presentation (Hedges *et al.* 2005).

A number of plasma proteins termed 'acute phase proteins' including complement and C-reactive protein (CRP) show a dramatic increase early in infection (Roitt *et al.* 2001). Complement is an important serum protein as it can be non-specific and assist innate immunity or target pathogens for phagocytosis, killing and presentation to the adaptive immune system.

Although innate immunity has been regarded as a non-specific system, investigations have shown a higher degree of specificity for this immune response (Akira and Takeda 2004). Toll-like Receptors (TLRs), described for their role in insect innate immunity against fungal infections (Lemaitre *et al.* 1996), have been shown to play a crucial role in early host defence against pathogens. TLRs, expressed primarily on macrophages and dendritic cells (DCs), recognize PAMPS and control the activation of these cells. Therefore, they have been described as linking innate recognition of non-self with the induction of adaptive immunity (Akira and Takeda 2004). In contrast, acquired immune



responses are slower and mediated by T and B cells. Both cells express highly diverse antigen receptors that are generated through DNA rearrangement, and respond to a wide range of antigens (Akira and Takeda 2004).

The adaptive immune response is organized around T and B lymphocytes. Since each lymphocyte displays a single kind of antigen receptor, the lymphocyte population is extremely diverse with an increased probability of recognizing antigen. Antigen recognition is a key to the development of an effective adaptive response. Antigen presenting cells (APCs) are seen as the interface between the innate and adaptive immune systems. They carry MHC class II molecules, which are important in presenting antigens to CD4<sup>+</sup> T helper (Th) cells. Antigens in association with MHC I (found on all nucleated cells) alert CD8<sup>+</sup> cytotoxic T cells to kill the infected cell (Roitt *et al.* 2001). Following interaction with APCs, CD4<sup>+</sup> cells are activated and stimulate mononuclear phagocytes to increase their killing activity, and B cells to synthesize antigen specific antibodies. Appropriate immunological systems are effective against different types of infection. Intracellular pathogens induce a cellular immune response characterized by the Th1 subset of CD4<sup>+</sup> T cells and cytotoxic CD8<sup>+</sup> T cells. In contrast, extracellular pathogens and allergens induce a humoral immune response, characterized by the Th2 subset of CD4<sup>+</sup> T cells.

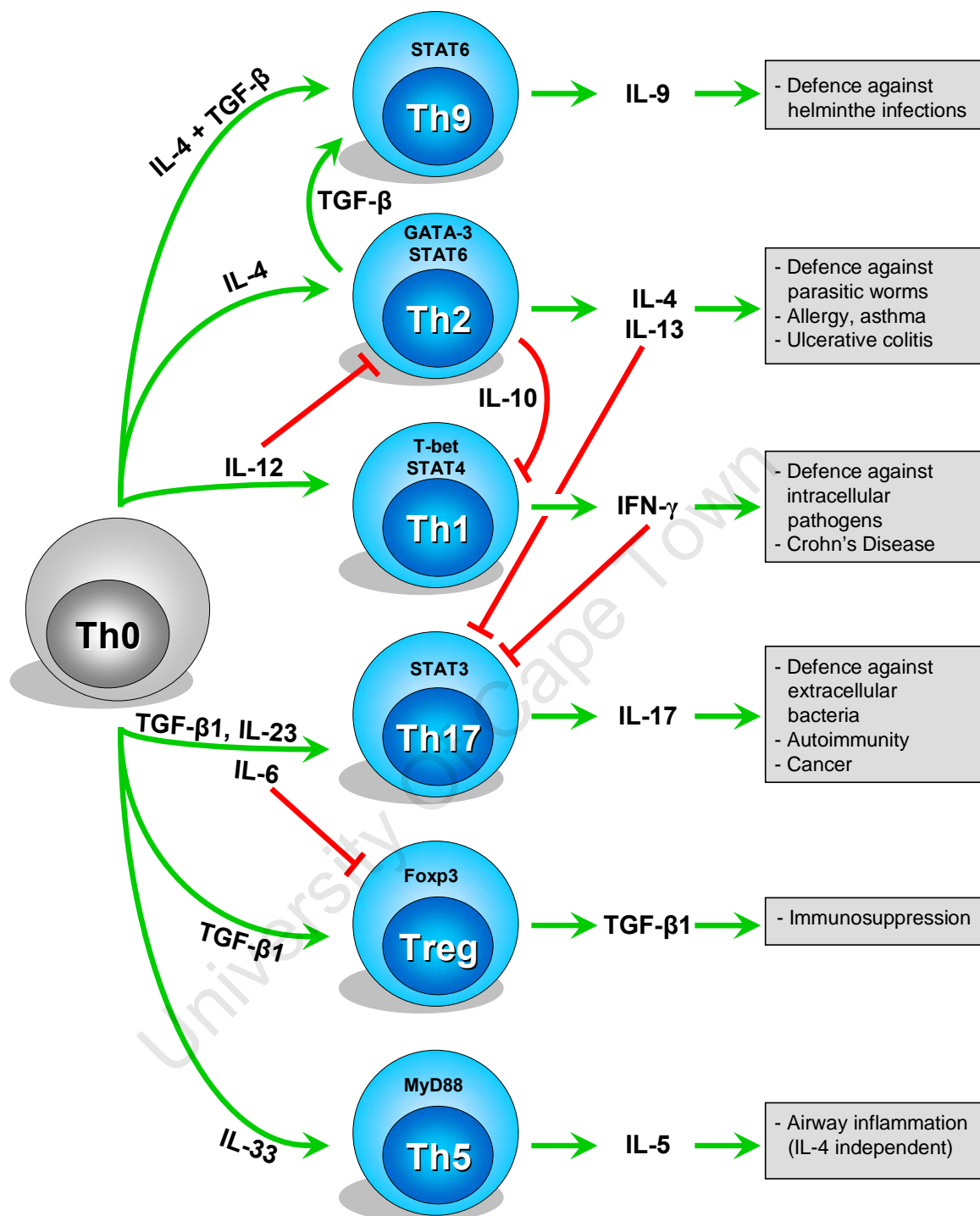
### **1.1.1. Cytokines**

Cytokines are regulatory proteins secreted by a variety of immune system cells. Most cytokines are low molecular weight polypeptides or glycoproteins, which can be induced or constitutive homeostatic. Cytokine production is transient and the action radius is short (Vilcek 1998). They have an autocrine function, acting on cells that produce them; or a paracrine function, acting on surrounding cells (Roitt *et al.* 2001). The actions of cytokines are pleiotropic; which stimulate or inhibit the differentiation, proliferation or function of immune cells and modulate inflammatory responses (Vilcek 1998). One cytokine can be produced by a variety of cell types and may often have overlapping functions on effector cells (Brombacher 2000), whereas a cytokine cascade may result in the increase/decrease of other cytokines. Furthermore, gene knockout mouse strains provide an essential tool in the analysis of cytokine function. However, compensatory mechanisms not normally present, may be activated in the absence of a gene (Vilcek 1998).

### 1.1.2. *T cell differentiation*

The differentiation of T helper cells into Th1 or Th2 subsets in response to infection and allergens plays an important role either in conferring immunity or mediating tissue damage. Which T cell subset gains predominance in an immune response depends on; (i) the type of antigen presenting cell, (ii) co-stimulatory molecules, (iii) the nature and dose of parasite and (iv) the immediate cytokine environment experienced at the time of antigen presentation (Onah and Nawa 2000). The cytokine IL-12 drives CD4<sup>+</sup> T cell differentiation into Th1 cells and induces IFN- $\gamma$  and IL-2 cytokine release (Figure 1). In general, Th1 responses are generated against intracellular pathogens driving protective IgG2a and complement-fixing antibodies, macrophage activation, antibody dependent cell-mediated cytotoxicity and delayed-type hypersensitivity. Th1 differentiation is initiated by signalling through; the T cell receptor (TCR), STAT1 associated cytokine receptors and IL-12 (Weaver *et al.* 2006). STAT1 signalling upregulates the transcription factor T-bet (Mullen *et al.* 2001), which stimulates IFN- $\gamma$  gene expression and upregulates the IL-12 receptor (IL-12R $\beta$ 2). Signalling through STAT4 via the IL-12R $\beta$ 2 stimulates expression of IFN- $\gamma$  and the IL-18 receptor, therefore creating a positive feedback.

A Th2 response driven by IL-4 is necessary for the elimination of helminth infections (Urban *et al.* 1991; Kopf *et al.* 1993; Svetic *et al.* 1993; Finkelman *et al.* 1997). In allergic reactions on the other hand, the body develops an exaggerated Th2 response to seemingly harmless antigens in genetically predisposed individuals which could lead to anaphylactic shock and death (Holgate 1999) (Figure 1). Th2 differentiation is driven by TCR and IL-4 receptor alpha (IL-4R $\alpha$ ) signalling via the STAT6 pathway. Together these signals upregulate expression of GATA-3 (Ferber *et al.* 1999). Th1 and Th2 responses counter regulate each other. STAT1 downregulates Th2 associated GATA-3; similarly STAT6 suppresses Th1 development by blocking IL-12R $\beta$ 2 expression (Weaver *et al.* 2006). Furthermore, IL-10 downregulates Th1 polarization, while IL-12 suppresses Th2 polarization.



**Figure 1: T-helper cell differentiation.**

Th1 and Th2 cells are derived from naïve Th0 cells. Th1 differentiation is prompted by IL-12 and IL-18 (from dendritic cells) and Th2 prompted by IL-4 (from NK, B cells, basophils and  $\gamma\delta$  cells). TGF- $\beta$  in combination with IL-4 drives the differentiation of Th9 cells directly or 'reprograms' Th2 cells to switch to IL-9 production. An inflammatory response mediated by TGF- $\beta$ 1 induces Th17 differentiation and a regulatory T cell response. Th5 cells are generated from naïve CD4<sup>+</sup> T cells by IL-33 in the presence of antigen. Products of Th1 and Th2 cells inhibit Th17 differentiation. Red arrows represent cross regulatory effects. Illustration adapted from previous publications (Brombacher 2000; Roitt *et al.* 2001; Tato and O'Shea 2006; Kurowska-Stolarska *et al.* 2008; Veldhoen *et al.* 2008).

Although the Th1/Th2 paradigm is widely accepted, it is clear that it does not cover all inflammatory responses or autoimmune diseases. Publications have shown a subset of CD4<sup>+</sup> IL-17-producing cells (Th17), which are stimulated by IL-6, TGF- $\beta$ 1 and IL-23 and distinct from Th1 or Th2 cells (Bettelli *et al.* 2006; Mangan *et al.* 2006). These Th17 cells are involved in immunity to extracellular bacteria but also mediate autoimmune disease (Figure 1). In contrast, IL-6 inhibits the generation of TGF- $\beta$ 1-stimulated CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells. Furthermore, a recent publication showed that TGF- $\beta$  ‘reprograms’ Th2 cells to lose their characteristic profile and secrete IL-9 instead. With the combination of IL-4, TGF- $\beta$  can drive the differentiation of cells producing IL-9 directly. This differentiation was shown to be dependent on STAT6 signalling. These cells are distinct from other CD4<sup>+</sup> T cell subsets and have been described as Th9 cells (Veldhoen *et al.* 2008). A role has also been described for IL-33; signalling via the ST2 pathway and MyD88, which induces CD4<sup>+</sup> T cells to secrete IL-5 independently of IL-4 or STAT6. IL-33 administration induces IL-5 producing ‘Th5’ cells and exacerbates allergen-induced airway inflammation in wild type and IL-4 knockout mice (Schmitz *et al.* 2005; Kurowska-Stolarska *et al.* 2008).

The T cell paradigm, as illustrated in Figure 1, can therefore be summarized as; differentiation into Th1 by IFN- $\gamma$ , Th2 by IL-4, Th17 by IL-23/TGF- $\beta$ 1/IL-6 and Treg by TGF- $\beta$ 1. However, the possibility of other T helper subsets such as Th5 and Th9 should not be excluded. It is unclear how many more effector T cell subsets there may be and what their role would be in controlling or causing disease. These results illustrate another exciting layer of complexity to the cytokine network induced by infection, opening new possibilities for the development of treatments.

## **1.2. Interleukin-4 and Interleukin-13**

### **1.2.1. Interleukin-4**

Mouse IL-4 is a glycoprotein with a molecular weight of 14-19 kDa (Yokota *et al.* 1986), localized on chromosome 11 together with genes for IL-5 and IL-13 (Morgan *et al.* 1992). The human IL-4 gene consists of four exons and three introns spanning 10kb (Arai *et al.* 1989). Innate sources of IL-4, such as basophils (Min *et al.* 2004), mast cells (Plaut *et al.* 1989),  $\gamma\delta$  T cells (Ferrick *et al.* 1995), NK1.1<sup>+</sup> T cells (Yoshimoto and Paul 1994), eosinophils (Sabin *et al.* 1996) and conventional T cells (Launois *et al.* 1995; Noben-Trauth *et al.* 2000) have been shown to initiate Th2 differentiation. However, IL-

4-independent Th2 differentiation has also been described (Noben-Trauth *et al.* 1997; Brombacher 2000; Jankovic *et al.* 2000; Mohrs *et al.* 2000; Ritz *et al.* 2002; Cunningham *et al.* 2004). Furthermore, IL-4 has been shown not to be essential for Th2 differentiation, as demonstrated in IL-4R $\alpha$  and STAT6-deficient mice that are able to generate sufficient numbers of Th2 cells in response to nematodes (Noben-Trauth *et al.* 1997; Finkelman *et al.* 2000). IL-4 increases the expression of genes such as the low affinity IgE receptor (CD23) (Defrance *et al.* 1987), IL-4R (Ohara and Paul 1987), MHC class II (Noelle *et al.* 1984), CD80 and CD86 (Stack *et al.* 1994). IL-4 also induces isotype switching to IgE and IgG1 in activated B cells while suppressing the synthesis of IgM, IgG2a and IgG2b (Vitetta *et al.* 1985; Coffman *et al.* 1986; Snapper *et al.* 1988).

### **1.2.2. Interleukin-13**

IL-13 is a 10-14 kDa immunoregulatory cytokine first described as a protein preferentially produced by activated mouse Th2 cells (Brown *et al.* 1989; Minty *et al.* 1993; Hershey 2003). Mouse IL-13 is localized on chromosome 11 together with genes for IL-3, IL-4, IL-5 and GM-CSF. The human IL-13 gene consists of four exons and three introns, localized 12 kb upstream of the gene encoding IL-4 on chromosome 5q31 (McKenzie *et al.* 1993; Smirnov *et al.* 1995). IL-13 is produced by T cells, NK T cells, mast cells, basophils, dendritic cells and NK cells (McKenzie *et al.* 1993; de Saint-Vis *et al.* 1998; Hoshino *et al.* 1999; Heller *et al.* 2005). IL-13 has distinct functions on a wide variety of cell types with diverse biological activities. A review by Thomas Wynn summarizes the effector functions of IL-13 (Wynn 2003). Essentially, IL-13 regulates gastrointestinal parasite expulsion, first shown in studies with *Nippostrongylus brasiliensis* (McKenzie *et al.* 1998; Urban *et al.* 1998) and intracellular parasitism in BALB/c mice susceptible to *Leishmania major* (Noben-Trauth *et al.* 1999). Furthermore, IL-13 regulates various aspects of inflammatory diseases of the lung shown in disease models of pulmonary inflammation (Chiaramonte *et al.* 1999; Blease *et al.* 2001), asthma (Grunig *et al.* 1998; Wills-Karp *et al.* 1998; Zhu *et al.* 1999) and anaphylaxis (Fallon *et al.* 2001). Functionally, IL-13 promotes proliferation of B cells, class switching to IgG4 and IgE, induces the expression of surface antigens (CD23 and MHCII), inhibits pro-inflammatory mediators by monocytes and macrophages, effects eosinophil function and activates mast cells (de Vries 1998; Oettgen and Geha 2001; Hershey 2003).

### ***1.2.3. IL-4 and IL-13 receptor complexes.***

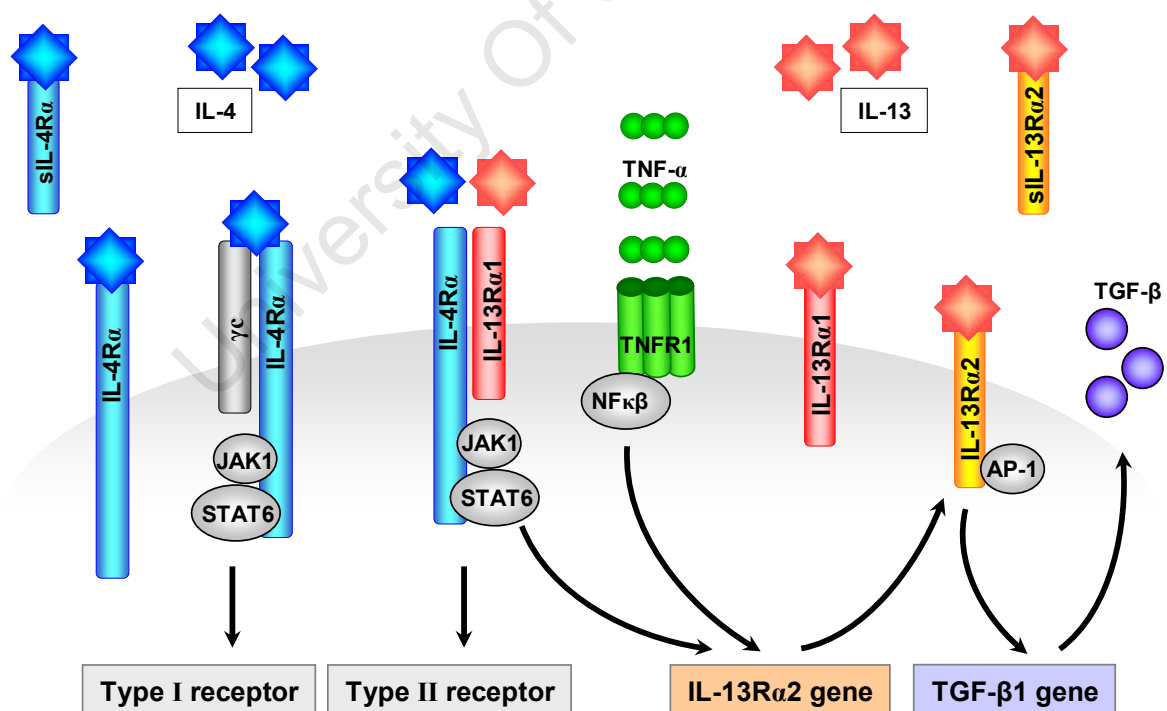
Both IL-4 and IL-13 cytokines use the IL-4R $\alpha$  chain as a component of their receptors (Figure 2). This was shown in mice treated with anti-IL-4R $\alpha$  antibodies or IL-4 antagonists (Aversa *et al.* 1993), which specifically blocked responses of both IL-4 and IL-13 (Aversa *et al.* 1993; Zurawski *et al.* 1993; Zurawski *et al.* 1995; Hilton *et al.* 1996). The IL-4R $\alpha$  consists of a 140-kDa IL-4R $\alpha$  chain which is a component of both the type I and type II IL-4 receptors. The IL-4R $\alpha$  is expressed in relatively low numbers on numerous cell types (Ohara and Paul 1987; Lowenthal *et al.* 1988; Nelms *et al.* 1999). The type I IL-4 receptor results from association of IL-4R $\alpha$  with the gamma common ( $\gamma$ c) chain, which is also a component of the receptors for IL-2, IL-7, IL-9, IL-15, and IL-21 (Leonard and Lin 2000; Hershey 2003). The type II IL-4/IL13 receptor results from association of IL-4R $\alpha$  with IL-13R $\alpha$ 1.

The type II receptor is composed of the IL-4R $\alpha$  chain and the 65-70 kDa IL-13R $\alpha$ 1 chain and serves as an alternative receptor for IL-4 (Figure 2). By itself, IL-13R $\alpha$ 1 binds IL-13 with low affinity but when paired with IL-4R $\alpha$ , it binds IL-13 with high affinity and forms a functional unit that signals (Miloux *et al.* 1997; Hershey 2003). IL-13R $\alpha$ 1 is expressed on many cell types including; B cells, monocytes, macrophages, basophils, mast cells and endothelial cells with the exception of human or mouse T cells (Gauchat *et al.* 1997; Graber *et al.* 1998; Hershey 2003). Although IL-13 signals via the IL-13R $\alpha$ 1, it has a higher binding affinity to the  $\alpha$ 2 chain of the IL-13 receptor (IL-13R $\alpha$ 2), which has previously been considered as a decoy receptor for IL-13 with no signal transduction. IL-13R $\alpha$ 2 is a 55-60 kDa protein closely related to IL-13R $\alpha$ 1 except that the cytoplasmic domain has no signalling motifs or binding sequences for signalling molecules (Colotta *et al.* 1994; Donaldson *et al.* 1998). However, recent publications have highlighted a possible signalling pathway for IL-13 through the IL-13R $\alpha$ 2. IL-13 signalling through the IL-13R $\alpha$ 2 was shown to be involved in the induction of TGF- $\beta$ 1 production or mediating fibrosis in a chronic mouse model of Crohn's disease (Fichtner-Feigl *et al.* 2006; Fichtner-Feigl *et al.* 2008). These studies suggest that IL-13R $\alpha$ 2 signalling could be an important therapeutic target for the prevention of inflammatory diseases.

In addition to cell surface receptors, soluble forms of both IL-4R $\alpha$  and IL-13R $\alpha$ 2 exist and are suggested to bind IL-4 and IL-13 with high affinity as non-signalling monomers. Therefore, the soluble receptors can act as competitive inhibitors of IL-4 and IL-13 and regulate their activity (Mosley *et al.* 1989; Zhang *et al.* 1997).

### 1.2.4. Mechanisms of IL-4 and IL-13 signalling

IL-4 and IL-13 not only share common subunits, they also share common signalling pathways (Figure 2). The components of both receptor complexes are associated with Janus kinases (JAK). JAK1 has been proposed to associate with the IL-4R $\alpha$  chain (Yin *et al.* 1994),  $\gamma$ c with JAK3 (Russell *et al.* 1994; Nelms *et al.* 1999) and IL-13R $\alpha$ 1 with JAK2 (Murata *et al.* 1996). The signal transducer and activator of transcription 6 (STAT6) is recruited to the phosphorylated IL-4R $\alpha$  where it also becomes phosphorylated by JAKs (Nelms *et al.* 1999). Studies using STAT6-deficient mice have determined that IL-13 signalling uses the JAK/STAT6 pathway (Takeda *et al.* 1996). Regulatory phosphatases (for negative regulation of signalling) and insulin receptor substrate (IRS) are other groups of signalling molecules recruited to the IL-4R $\alpha$  chain (Nelms *et al.* 1999). Until recently the IL-13R $\alpha$ 2, which binds IL-13 with high affinity, was thought to relay no signal (Kawakami *et al.* 2001). Fichtner-Feigl *et al.* have shown that IL-13 signalling through IL-13R $\alpha$ 1/IL-4R $\alpha$  together with TNF- $\alpha$  signalling through TNFR1, up regulates IL-13R $\alpha$ 2 surface expression on macrophages. IL-13 binding this receptor activates AP-1 to induce secretion of TGF- $\beta$ .



**Figure 2: IL-4 and IL-13 receptor complexes.**

IL-4 interacts with the type I (IL-4R $\alpha$  and  $\gamma$ c) and type II (IL-4R $\alpha$  and IL-13R $\alpha$ 1) receptor complexes. These receptors then signal through the JAK1/STAT6 pathway. IL-13 interacts with the type II receptor complex and with IL-13R $\alpha$ 2. IL-13 also induces production of TGF- $\beta$  in macrophages in two steps. IL-13R $\alpha$ 2 surface expression is upregulated in response to a combination of IL-13 and TNF- $\alpha$ . IL-13 binds to IL-13R $\alpha$ 2 with high affinity and activates AP-1 to induce secretion of TGF- $\beta$ . Illustration adapted from previous publications (Brombacher 2000; Hershey 2003; MacDonald 2006).

### **1.3. Inflammatory Bowel Disease**

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic, immunologically mediated disorders collectively referred to as human inflammatory bowel disease (IBD). For UC long term inflammation and destruction eventually leads to the removal of the large intestine or colectomy. Although the incidence of both CD and UC is beginning to stabilize in high-incidence areas, cases are increasing in low-incidence areas including, southern Europe, Asia and parts of the developing world (Loftus 2004). UC and CD share many clinical and pathological characteristics, but they have immunological and disease processes which are distinct for each disease (Podolsky 2002; Bouma and Strober 2003; Targan and Karp 2005; Sartor 2006a). UC can be distinguished from CD by the nature of inflammatory changes in the gut. UC is restricted to the superficial epithelial layer of the colon, while CD is found throughout the intestine affecting multiple layers (Bouma and Strober 2003). Although the pathogenesis of UC and CD is unclear, it is suggested that the intestinal mucosal immune system is disrupted leading to a loss of tolerance to commensal bacterial in genetically predisposed individuals (Podolsky 2002; Bouma and Strober 2003). Environmental triggers are also necessary to initiate or reactivate disease expression. Defects in bacterial antigen sampling leads to an inappropriate immune response with activation of dendritic cells and an overly aggressive acquired T cell immune response. Immunological events driven by a cascade of cytokines finally result in tissue damage including; loss of epithelial and goblet cells, oedema and ulceration (Podolsky 2002; Sartor 2006a).

#### ***1.3.1. Classification and features of inflammatory bowel disease***

Ulcerative colitis and Crohn's disease have many similarities but can be distinguished by clinical, pathological and immunological features. However, in as many as 10 percent of patients with IBD that is limited to the colon, it may not be possible to distinguish UC from CD initially. These patients are considered to have indeterminate colitis (Podolsky 2002; Bouma and Strober 2003). Table 1 summarizes some of the symptoms and features used to diagnose human IBD (adapted from Podolsky 2002; Bouma and Strober 2003).

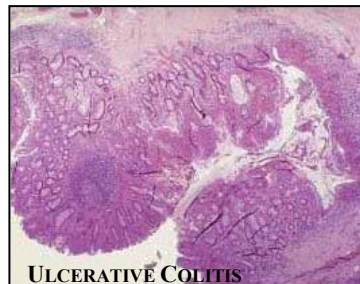


**TABLE 1: KEY FEATURES OF MAJOR FORMS OF INFLAMMATORY BOWEL DISEASE**

FEATURE	ULCERATIVE COLITIS	CROHN'S DISEASE
<b>Clinical features</b>		
Fever	Fairly common	Common
Abdominal pain	Varies	Common
Diarrhoea	Very common	Fairly common
Rectal bleeding	Very common	Fairly common
Weight loss	Fairly common	Common
Signs of malnutrition	Fairly common	Common
Perianal disease	Absent	Fairly common
Abdominal mass	Absent	Common
<b>Site</b>		
Colon	Exclusively	2/3 of patients
Ileum	Never	2/3 of patients
Jejunum	Never	Infrequent
Stomach or duodenum	Never	Infrequent
Oesophagus	Never	Infrequent
<b>Intestinal complications</b>		
Stricture	Unknown	Common
Fistulas	Absent	Fairly common
Cancer	Common	Fairly common
<b>Endoscopic finding</b>		
Friability	Very common	Fairly common
Aphthous and linear ulcers	Absent	Common
Cobblestone appearance	Absent	Common
Pseudopolyps	Common	Fairly common
Rectal involvement	Very common	Fairly common
<b>Radiologic findings</b>		
Distribution	Continuous	Discontinuous, segmented
Ulceration	Fine, superficial	Deep, with submucosal extension
Fissures	Absent	Common
Strictures and fistulas	Rare	Common
<b>Histology</b>		
	Superficial layers of bowel wall	All layers of bowel wall
	Infiltration of lymphocytes and neutrophils	Infiltration of lymphocytes and macrophages
	Loss of goblet cells	Presence of granulomas
	Ulceration	Ulceration
	Crypt abscesses	Submucosal fibrosis

(Left) Histology section of a patient with UC showing mucosal inflammation and erosion.

(Right) Histology section of a patient with CD showing inflammation extending the full thickness of the wall from the mucosa to the serosa. Granulomas (G) can be seen towards the serosal surface.



### ***1.3.2. Factors contributing to ulcerative colitis***

#### **Genetics**

Although evidence suggests UC is linked to genetic factors, there is no single gene responsible for the disease. Different genetic abnormalities can lead to similar clinical features of intestinal inflammation. Several genes with increased association among patients with UC have been demonstrated. The MHC class II region on chromosome 6 (known as IBD3) has been associated with UC (Toyoda *et al.* 1993; Satsangi *et al.* 1996; Targan and Karp 2005) as have IBD2 and IBD6 loci. Recently, researchers performed a genome-wide study on UC patients to identify other genes involved in the disease. Genes of interest on chromosome 1p36 were, the ring finger protein 186 (RNF186), OTU domain containing 3 (OTUD3), and phospholipase A2 group IIE (PLA2G2E). Genes of interest on chromosome 12q15 were the IFN- $\gamma$ , IL-26 and IL-22 genes playing a role in inflammation and the immune response (Imielinski *et al.* 2009). Furthermore, another recent study has shown that a missense error in the membrane-bound transcription factor peptidase site 1 (S1P)-encoding gene (Mbtps1) causes enhanced susceptibility to dextran sodium sulfate (DSS), a mouse model of UC (Brandl *et al.* 2009).

#### **Commensal microbial stimulants**

Although genetic background is proving to be an important requisite for UC, environmental factors such as commensal bacteria and persistent infection also influence genetic susceptibility. In contrast to normal patients, T cells from patients with UC or CD proliferate in response to both autologous and heterologous faecal bacteria (Duchmann *et al.* 1995; Sartor 2006b). Furthermore, the presence of bacterial overgrowth and relative effectiveness of antibiotic treatment suggests that microbes may be responsible for inflammation in the genetically predisposed mucosa (Targan and Karp 2005). In experimental colitis, mice are generally protected from disease when kept in a germ-free environment, suggesting that normal flora plays a role in initiating inflammation either by functioning as adjuvants or antigens. Adjuvants are responsible for activating innate immune responses including APCs and antigens which stimulate the clonal expansion of T cells via the T cell receptor (Sartor 1997; Podolsky 2002; Bouma and Strober 2003).

### ***1.3.3. Mouse models of Inflammatory Bowel Disease***

Animal models of intestinal inflammation are essential in our understanding of IBD. Although animal models do not represent the complexity of human disease they allow us

to study pathological mechanisms and the effect of emerging therapeutic strategies. Many different factors induce intestinal inflammation in mice but the final common pathway eventually resolves into a Th1 or Th2 response (Elson *et al.* 1995; Strober *et al.* 2002; Elson *et al.* 2005; Wirtz and Neurath 2007). Table 2 lists a few animal models used to study Th1 and Th2 mediated mucosal inflammation (adapted from Strober *et al.* 2002). The Th1 pathway has been shown to be predominant in IBD with most models developing an excessive IFN- $\gamma$  response. This Th1 bias could be due to inflammation being driven by antigens from the intestinal microflora, which initiates innate immune responses mediated by TLRs. TLRs generally activate NF- $\kappa$ B which leads to IL-12 production and a polarized Th1 response (Takeda *et al.* 2003; Strober and Fuss 2006). Although, CD4<sup>+</sup> Th1 cells mediate disease in most IBD models and resemble Crohn's disease, CD4<sup>+</sup> Th2-mediated colitis models resembling ulcerative colitis have also been described (see table 2).

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**TABLE 2:** T CELL-MEDIATED MOUSE MODELS OF MUCOSAL INFLAMMATION

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Th1 MODELS	Th2 MODELS
TNBS colitis	TCR- $\alpha$ chain deficiency
SCID-transfer colitis	Oxazolone colitis
TCR Tg mice with lymphopenia	WASP deficiency
IL-10 deficiency colitis	DSS colitis *
IL-10 signalling defects (CRF2-4 deficiency)	TNBS colitis in BALB/c mice *
Tg $\epsilon$ 26 mice	
TNF <sup>ARE</sup> mice (TNF- $\alpha$ overproduction)	
C <sub>3</sub> H/HeJBir mice	
Gi2 $\alpha$ -deficient mice	
Samp1/Yit mice	
T-bet Tg mice	
STAT4 Tg mice	
TGF- $\beta$ RII dominant-negative Tg mice	
HLA-B27 Tg rats	
<i>Mdrla</i> -deficient mice	
DSS colitis	
IL-7 Tg mice	

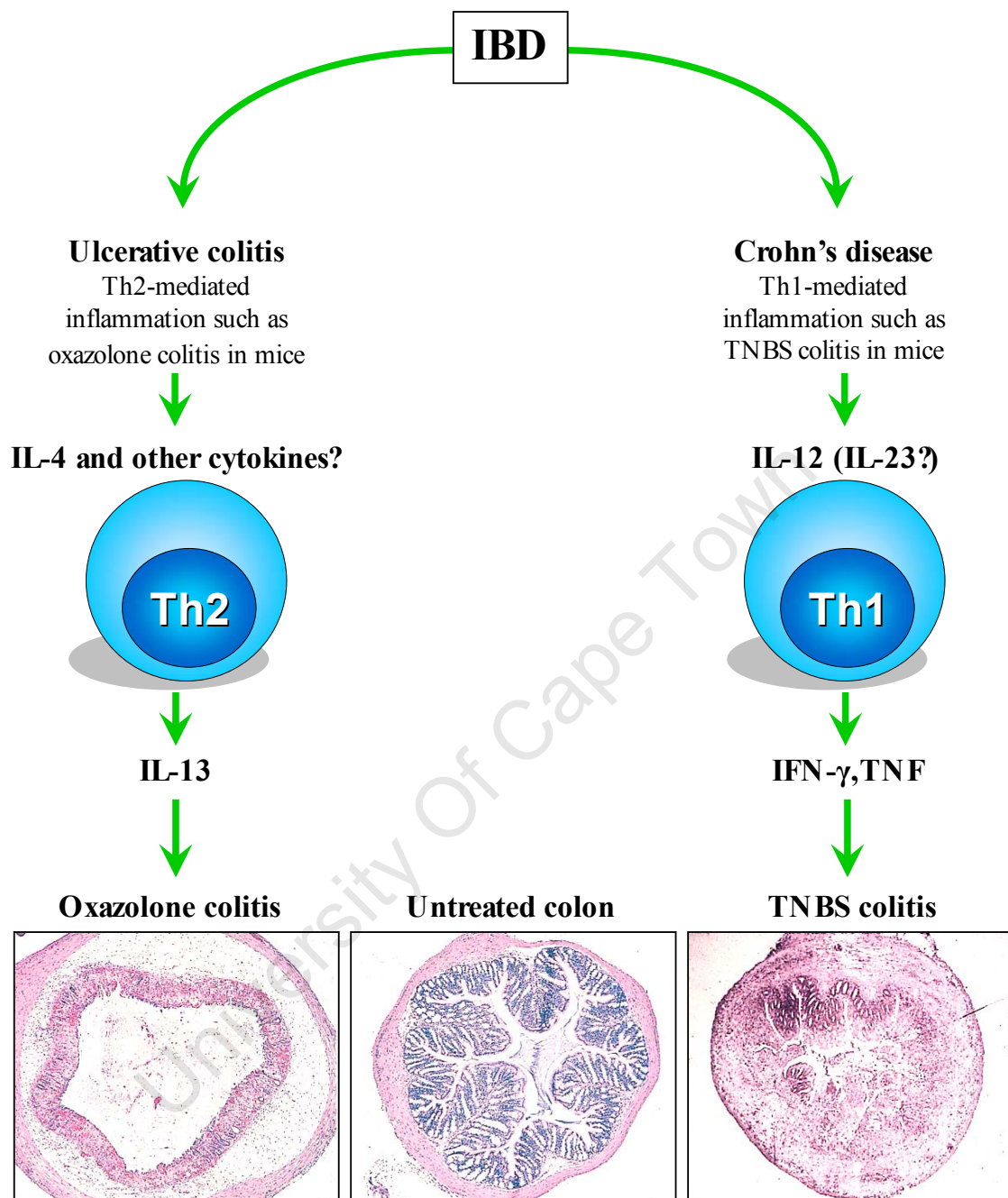
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\* Mixed response but initially Th1, later Th2

It has been shown that low affinity interactions between antigen and TCR favour Th2 differentiation, therefore the small amounts of IL-4 required to drive a Th2 response are produced (Pfeiffer *et al.* 1995; Guo *et al.* 2004; Strober and Fuss 2006). Previous studies using hapten-induced mouse models of colitis have shown that the genetic background of a mouse influences the outcome of T cell responses. Trinitrobenzene sulphonic acid (TNBS)-induced colitis mediates a Th1-driven inflammation which resembles CD in SJL/J mice, but a mixed Th1/Th2 disease in BALB/c mice (Neurath *et al.* 1995; Dohi *et al.* 1999). Oxazolone-induced colitis mediates a Th2-driven inflammation which resembles UC in SJL/J, BALB/c and C57BL/10 mice (Baird *et al.* 1998; Heller *et al.* 2002; Kojima *et al.* 2004; Fichtner-Feigl *et al.* 2006; Weigmann *et al.* 2008). However, C57BL/6 mice have a tendency towards Th1 immune responses (Charles *et al.* 1999) and require pre-sensitization with oxazolone to induce a Th2 response (Iijima *et al.* 2004). Chemically induced models of colitis are widely accepted and used as IBD animal models and although they have limitations, they resemble important histological and immunological aspects of human IBD (Wirtz and Neurath 2007) (see Figure 3). TNBS and oxazolone are both covalently reactive reagents which are believed to induce a T cell-mediated response against hapten-modified luminal antigens. Dextran sodium sulphate (DSS) is a chemical directly toxic to colonic epithelial cells of the basal crypts (Morris *et al.* 1989; Okayasu *et al.* 1990; Yamada *et al.* 1992; Baird *et al.* 1998; Wirtz and Neurath 2007).

### **TNBS colitis**

As with oxazolone, TNBS dissolved in ethanol with or without skin pre-sensitization induces colitis in susceptible mouse strains. Ethanol is required to break the mucosal barrier and TNBS or oxazolone is thought to haptenize colonic proteins making them immunogenic to the host (Neurath *et al.* 1995). TNBS induces an IL-12-mediated Th1-cell response in SJL/J mice and inflammation that resembles CD (see Figure 3). Disease parameters include; inflammation affecting the entire colon, infiltration of lymphocytes and macrophages, thickening of the colon wall, ulceration, loss of goblet cells and the presence of granulomas (Neurath *et al.* 1995; Bouma and Strober 2003). Antibodies blocking IL-12 not only prevents TNBS colitis but can also successfully treat established colitis (Neurath *et al.* 1995). Furthermore, a crucial role for TNF- $\alpha$  in CD was confirmed using TNF- $\alpha$  knockout mice which were protected from TNBS colitis. In contrast, mice over-expressing TNF- $\alpha$  developed severe colitis in response to TNBS treatment (Neurath *et al.* 1997).



**Figure 3: Th1 and Th2-mediated colitis.**

Trinitrobenzene sulphonic acid (TNBS) and oxazolone give rise to different forms of colitis when administered intrarectally together with ethanol (an agent that temporarily decreases epithelial barrier function). These colitides are strain-specific and genetically determined. TNBS that is administered to SJL/J or C57BL/10 mice elicits a classical T helper (Th1)-cell response owing to IL-12-mediated production of IFN- $\gamma$  by CD4<sup>+</sup> T cells. The result is a transmural inflammation that resembles Crohn's disease. By contrast, oxazolone induces a Th2-cell response in SJL/J, C57BL/10 and BALB/c mice that is mediated by natural killer T cells that produce IL-13. The result is a superficial inflammation that resembles ulcerative colitis (adapted from Bouma and Strober 2003).

### **Oxazolone colitis**

Oxazolone colitis was first described by Boirivant and colleagues in 1998 and has since been widely accepted by other researchers as an IL-4/IL-13-mediated Th2 model for human ulcerative colitis (see Figure 3) (Boirivant *et al.* 1998). The model was established in highly susceptible SJL/J mice and then C57 background strains for the benefit of using genetically manipulated mice (Heller *et al.* 2002; Nieuwenhuis *et al.* 2002). Subsequently, oxazolone colitis has been successfully induced in BALB/c mice and the tissue damage (although transient) reflects that of human UC. Disease parameters include; weight loss, inflammation affecting the distal colon, infiltration of lymphocytes and granulocytes, patchy ulceration, loss of goblet cells, epithelial cell loss and oedema. The colitis is superficial as outer muscle layers show no evidence of inflammation (Kojima *et al.* 2004). Blocking IL-4 (Boirivant *et al.* 1998), IL-13 (Heller *et al.* 2002) or using IL-13 gene-deficient mice (Weigmann *et al.* 2008) has been shown to ameliorate or prevent disease induction, whereas antibodies against IL-12 cause severe pancolitis affecting both the distal and proximal colon (Boirivant *et al.* 1998).

### **DSS colitis**

Oral administration of DSS polymers has been shown to induce acute and chronic colitis in mice by affecting the integrity of the mucosal barrier. Early lesions occur mainly in the distal colon and are associated with bloody diarrhoea, ulcerations and granulocyte infiltration (Okayasu *et al.* 1990; Wirtz *et al.* 2007; Wirtz and Neurath 2007). SCID mice also develop acute colitis in response to DSS, indicating that T and B cells are not required. Therefore, the acute DSS model is useful to study innate immune mechanisms of colitis (Dieleman *et al.* 1994). Another study by Dielmann *et al.* showed that the chronic phase of DSS-induced colitis is characterized by both Th1 and Th2 cytokine profiles, suggesting that chronic immune activation is mediated by both populations of Th cells (Dieleman *et al.* 1998). When DSS is administered for several cycles, susceptible mouse strains develop chronic colitis and addition of the colon carcinogen azoxymethane (AOM) results in inflammation-associated colorectal cancer (Tanaka *et al.* 2003). As patients with UC have an increased risk of developing colon cancer (Jess *et al.* 2006) this model is useful to study the mechanisms linking inflammation to colon cancer (Wirtz and Neurath 2007).

#### ***1.3.4. Immune response in human ulcerative colitis***

The function of the mucosal immune system is to protect the epithelial barrier from invasion by microbes but avoid responding to commensal bacteria or food proteins. Tolerance in normal hosts is mediated by regulatory T cells, B lymphocytes, NKT cells and dendritic cells. Essentially, antigen presented to T cells with low levels of costimulation and immunoregulatory cytokines induce tolerance (Brandtzaeg 1996). Upon defective control mechanisms, an enhanced uptake of commensal bacteria adjuvants and antigens drive chronic intestinal inflammation resulting in IBD (Nagler-Anderson 2001; Sartor 2006a). This defect could be due to transient infection, ingested toxin or genetic susceptibility of the host. Finally, immunoregulatory defects or continued antigenic stimulation lead to pathogenic Th1 or Th2 immunological responses (Sartor 2006b).

In human UC the CD4<sup>+</sup> subset responsible for disease pathogenesis is not completely defined, but based on the histopathological resemblance to Th2-mediated mouse models of mucosal inflammation, pathology is accepted to be mediated by an atypical Th2 response (Boirivant *et al.* 1998; Mizoguchi *et al.* 1999; Elson *et al.* 2006). Increased production of Th2 cytokines, such as IL-5 and IL-13 have been found in lamina propria cells from UC patients, but IL-4 is not increased (Fuss *et al.* 1996; Heller *et al.* 2005). However, a study using the oxazolone mouse model shows IL-4 to be important in disease induction in SJL/J mice, with IL-13 acting later in disease progression and chronicity (Heller *et al.* 2002). Therefore, diagnosis of human UC might be after the IL-4 levels have been superseded by increased IL-13.

Due to the increased epithelial permeability with excessive antigen exposure, distorted B cell differentiation leads to an immunological imbalance and second line of mucosal defence. This includes the production of IgG antibodies which exhibit proinflammatory properties. Therefore, there is an increase in antigen penetrating the epithelial barrier, attraction of inflammatory cells and release of inflammatory mediators (Brandtzaeg *et al.* 2006). In UC patients, there is an increase in IgG antibodies and the cells which produce them (Hibi *et al.* 1990; Onuma *et al.* 2000). However, it is not certain if antibodies play a role in the pathology of UC or if B cells have a regulatory role rather than pathogenic (Mizoguchi *et al.* 1997; Sartor 2006a).

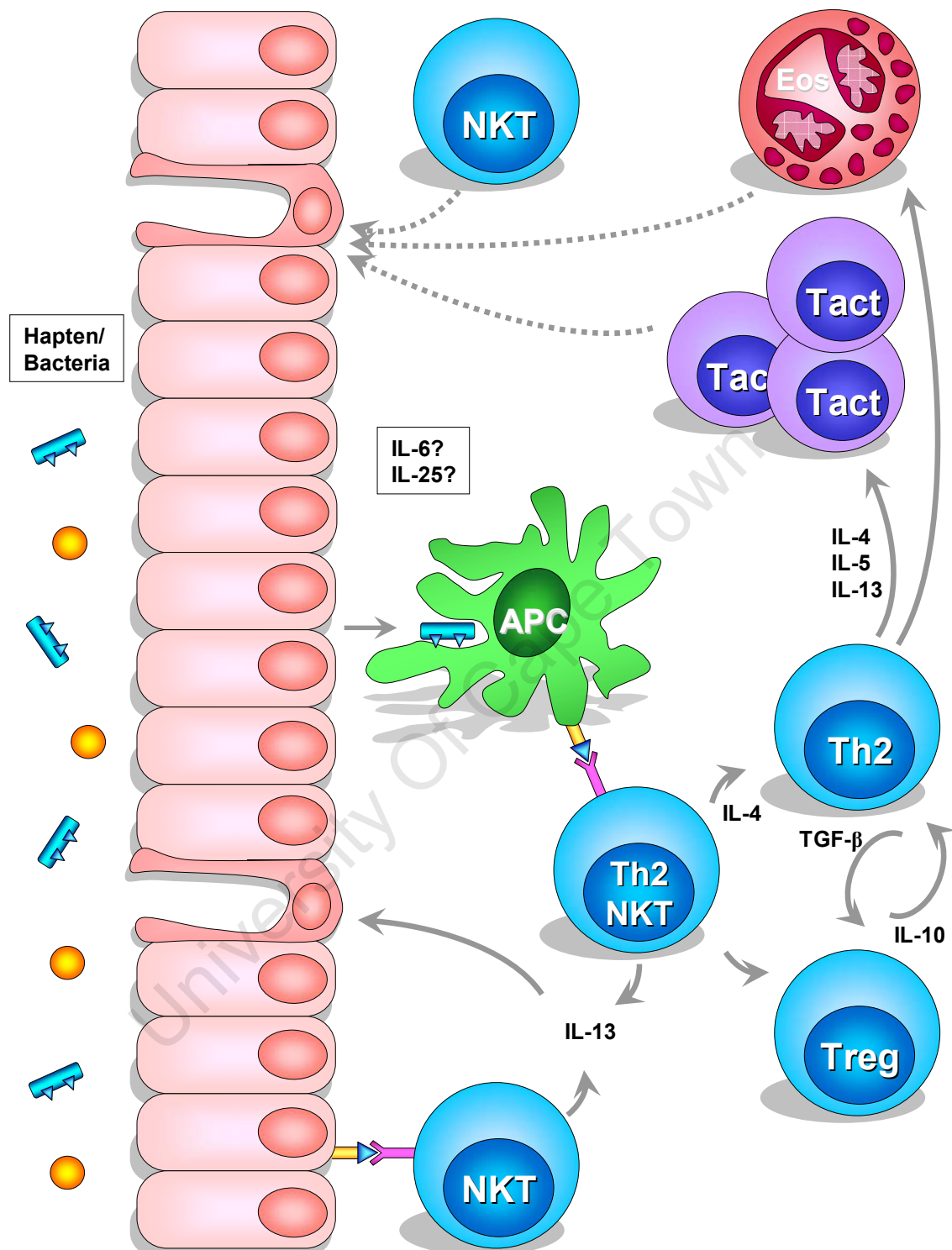
Finally, cytokine production initiates a cascade of inflammatory events which results in tissue damage. Although many aspects of this response remain to be understood, studies have highlighted a role for IL-13 acting directly on epithelial cells. Heller *et al.* show that IL-13 impairs epithelial barrier function by affecting epithelial apoptosis and tight junctions leading to the severe destructive inflammation seen in patients with UC (Heller *et al.* 2005).

### ***1.3.5. Animal models suggest immune mechanisms for human ulcerative colitis***

Mouse models of intestinal inflammation are valuable tools in understanding the pathogenesis of IBD in humans. Figure 4 illustrates some of the mechanisms underlying UC created by combining information from animal and human studies. The importance of Th2 cytokines was first demonstrated with studies showing that IL-4 deficiency or neutralizing IL-4 protected SJL/J mice from oxazolone colitis (Boirivant *et al.* 1998; Iijima *et al.* 2004). As mentioned above, IL-4 was shown to be important in initiating colitis but is superseded by IL-13 production in chronic oxazolone models. Further studies have suggested that IL-13 production by NKT cells is the main inducer of disease (Heller *et al.* 2002). Blocking IL-13 or depleting NKT cells protects mice from developing inflammation. The importance of NKT cells in oxazolone-induced colitis was also demonstrated in a study using mice deficient in the Epstein–Barr virus-induced gene 3 (EBI3). These mice exhibit normal numbers of naïve and mature CD4<sup>+</sup>, CD8<sup>+</sup> T cells and B cells but significantly reduced numbers of invariant NKT cells (iNKT). EBI3<sup>-/-</sup> mice were resistant to oxazolone-induced colitis with reduced IL-4 suggesting EBI3 to play a critical role in the induction of type 2 immune responses possibly mediated through iNKT cell function (Nieuwenhuis *et al.* 2002).

Another Th2 cytokine recently shown to be of interest in UC is IL-6. Essentially, the nuclear factor of activated T cells (NFATc2) is upregulated in human UC patients and oxazolone-treated mice. NFATc2 deficiency in mice suppresses oxazolone-induced colitis with reduced production of IL-6 and IL-13 by mucosal T cells. IL-6-deficient mice were protected from oxazolone colitis suggesting a regulatory role for NFATc2 in colitis in an IL-6-dependent manner (Weigmann *et al.* 2008). Furthermore, IL-25 has been shown to be important in controlling immunity in the gut and could prove interesting in UC studies (Owyang *et al.* 2006). IL-25 induces (i) epithelial cell hyperplasia (ii) mucus production and (iii) infiltration with granulocytes; associated with increased IL-4, IL-5 and IL-13 gene expression (Fort *et al.* 2001).





**Figure 4: Pathogenesis of ulcerative colitis.**

Commensal bacteria products stimulate epithelium or the mucosal immune system by penetration of the mucosal barrier. Activation of antigen presenting cells (APCs) or direct stimulation of NKT cells results in a type 2 response. NKT cells could have a cytotoxic effect on the epithelium directly or produce IL-13 which is toxic to epithelial cells. Recent studies have proposed roles for IL-6 and IL-25 in driving Th2 responses in UC (adapted from Podolsky 2002; Bouma and Strober 2003; Targan and Karp 2005; Owyang *et al.* 2006; Weigmann *et al.* 2008).

Considering the importance of IL-13 signalling in both human UC and mouse oxazolone colitis, studies are investigating the role of IL-13R $\alpha$ 2 (the high affinity receptor for IL-13) in colitis models (Fichtner-Feigl *et al.* 2006; Fichtner-Feigl *et al.* 2008). As the cytoplasmic region of murine IL-13R $\alpha$ 2 has no obvious signalling motif, it was previously thought to be a decoy receptor for IL-13 (Donaldson *et al.* 1998; Wynn 2003). Fichtner-Feigl *et al.* demonstrated the signalling pathway for IL-13R $\alpha$ 2 through AP-1 to induce TGF- $\beta$  production (see Figure 2). Signalling through IL-13R $\alpha$ 2 is dependent on TNF- $\alpha$ . Blocking TNF- $\alpha$  leads to reduced TGF- $\beta$  production in oxazolone-induced colitis with inflammation no longer limited to the distal colon (Fichtner-Feigl *et al.* 2006).

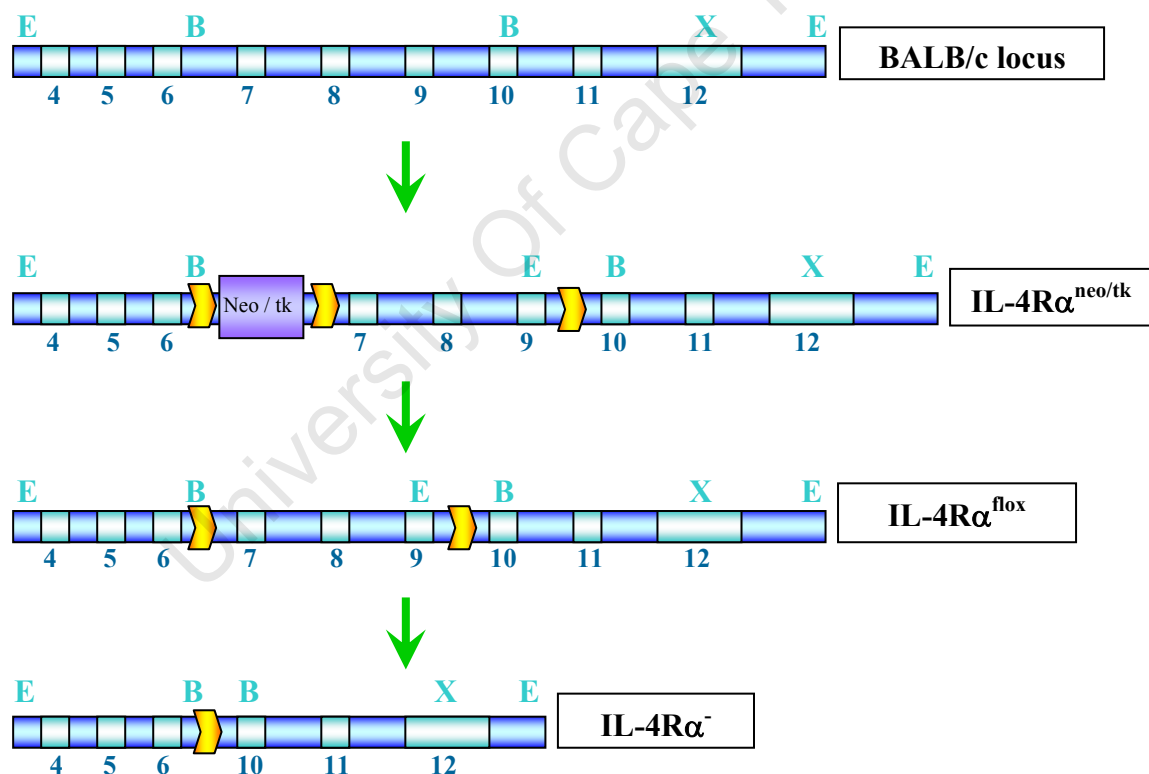
#### **1.4. Therapeutic strategy for ulcerative colitis**

Current treatment strategies for UC use broad anti-inflammatory drugs and are not specific for the disease. 5-Aminosalicylates (5-ASA) are bowel-specific anti-inflammatory drugs, considered a first-line therapy for mild to moderately active UC and have been in use since the forties (Dick *et al.* 1964; Sandborn *et al.* 2009). Other treatments include; corticosteroids, azathioprine/6-mercaptopurine (reduce lymphocyte numbers), intravenous cyclosporine (immunosuppressant) and colectomy (Bouma and Strober 2003). Animal models of disease provide vital information on the immune mechanisms involved in UC and could assist in finding a new approach to specific treatment strategies. Using the TNBS colitis mouse model resembling human CD, it was established that IL-12 driven Th1 cell responses were responsible for inducing inflammation. Anti-IL-12 antibodies successfully prevented and treated disease development in mice. Based on these studies, anti-IL-12 was used in a trial as a therapeutic agent in humans with CD, showing promising results (Mannon *et al.* 2004). UC has been defined as a Th2-mediated inflammation which leads to the possibility that Th2 cytokine inhibitors, such as IL-13-specific antibody, might be a useful target to treat this kind of IBD. Another cytokine of interest is IL-6, shown to be increased in human UC (Atreya *et al.* 2000). IL-6-deficient BALB/c mice are protected from oxazolone-induced colitis suggesting that this cytokine is essential in driving disease (Weigmann *et al.* 2008).

#### **1.5. Cell-specific gene targeting**

The IL-4R $\alpha$  is found on haematopoietic, endothelial, epithelial, muscle, fibroblast, hepatocyte and brain tissue (Nelms *et al.* 1999). In order to study the effects of IL-4 or IL-13 on specific cell types it is necessary to target the IL-4R $\alpha$  gene specifically, this is

accomplished by using Cre/*loxP* recombination (see Figure 5). Cyclization recombinase (Cre) inserted downstream of the promoter recognises a pair of *loxP* binding sites flanking the gene of interest (Exon7 through 9 of IL-4R $\alpha$ ). Cre-recombinase brings the two *loxP* sites together, removing the intervening DNA which encodes the transmembrane, soluble and extra cellular membrane proximal regions (Nagy 2000). To specifically target IL-4R $\alpha$  deletion on T cells (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>), Cre-recombinase expression was driven using the T cell-specific Lck promoter (Gu *et al.* 1994; Radwanska *et al.* 2007). Lck protein tyrosine is a kinase involved in the T cell signal transduction pathway (Janeway and Travers 1996). For novel B cell-specific IL-4R $\alpha$ -deficient mice (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>), Cre-recombinase was driven by the mb1 promoter. The mb1 gene encodes the Ig- $\alpha$  signaling subunit of the B cell antigen receptor (Hobeika *et al.* 2006).



**Figure 5: IL-4R $\alpha$  gene locus and targeted deletion.**

Introduction of a neo/tk selection cassette flanked by two *loxP* sites (yellow arrowhead), and one *loxP* site 5' of exon 10 resulted in the generation of the conditional "floxed" mouse after Cre-mediated recombination. An intercross between the floxed strain and Lck<sup>cre</sup>, or mb1<sup>cre</sup> knockin strain facilitated the generation of T or B cell-specific IL-4R $\alpha$ -deficient mice. Numbers indicate exons of the IL-4R $\alpha$ ; B, E and X, restriction sites for BamHI, EcoRI and XhoI (described by Herbert *et al.* 2004).

## 1.6. Objectives

- To establish a chemically-induced (oxazolone) mouse model of ulcerative colitis in BALB/c mice.
- To investigate the role of Th2 cytokines in oxazolone-induced colitis by using BALB/c mice deficient in genes for IL-4, IL-13 and IL-4R $\alpha$  or overexpressing IL-13.
- To determine the role of IL-4 responsive B cells and IgE production in oxazolone colitis.
- To determine the role of IL-4 responsive CD4<sup>+</sup> T cells in oxazolone colitis.

## 1.7. References

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## **Chapter 2: Experimental Section**

## **2.1 Experimental Study 1**

**IL-13 signals independently of IL-4 receptor-alpha to  
cause oxazolone-induced colitis**



### ABSTRACT

Ulcerative Colitis (UC) is a human Inflammatory Bowel Disease (IBD) associated with chronic inflammation of the gastrointestinal tract. Although UC is associated with a Th2/Type 2 immune response, current treatment strategies use broad anti-inflammatory drugs which are aspecific for the disease. A mouse model resembling UC is oxazolone-induced colitis. In this model interleukin (IL)-4 and IL-13 production are important pathological factors and neutralizing these cytokines prevents or ameliorates disease significantly. As many aspects of the mechanisms involving these Th2 cytokines in colitis remain undefined, we used mice deficient in IL-4/IL-13 or their shared receptor chain, IL-4 receptor-alpha (IL-4R $\alpha$ ), to further dissect their role in oxazolone-induced colitis.

Treatment of wild type BALB/c mice with oxazolone induced rapidly developing colitis confined to the distal colon with characteristics resembling human UC. Disease symptoms were exacerbated in IL-4R $\alpha$ <sup>-/-</sup> mice with increased weight loss, mortality, inflammation and immunopathological symptoms. This was in contrast to IL-4/IL-13 double-deficient mice which were protected from colitis. Removing IL-13 production from IL-4R $\alpha$ <sup>-/-</sup> mice, by using IL-4R $\alpha$ /IL-13 double-deficient mice, reversed the susceptible phenotype to protection. Together these data strongly suggest that IL-13 mediates susceptibility in an IL-4R $\alpha$  independent manner. Recent evidence shows that the IL-13 receptor-alpha 2 (IL-13R $\alpha$ 2), previously believed to be a decoy for IL-13 only, is able to transmit a signal via IL-13. Our results support this and may suggest that IL-13 signalling through IL-13R $\alpha$ 2 plays an important role in mediating disease in oxazolone-induced colitis.

## INTRODUCTION

Ulcerative colitis (UC) is a human Inflammatory Bowel Disease (IBD) associated with chronic inflammation of the gastrointestinal tract. Although the pathogenesis of UC is unclear, it is suggested that the intestinal mucosal immune system is disrupted leading to loss of tolerance to commensal bacteria in genetically predisposed individuals. UC in humans is believed to be a Th2-mediated disease (Podolsky 2002; Bouma and Strober 2003; Targan and Karp 2005). In mice, treatment with the haptening agent oxazolone (oxa) induces colitis limited to the distal half of the colon with histological features resembling human UC. Oxazolone colitis exhibits a Th2 profile in mice with increased levels of IL-4, IL-5 and IL-13 (Baird *et al.* 1998; Kojima *et al.* 2004; Wang *et al.* 2004). Previous studies highlight the importance of IL-4 and natural killer (NK) T cells producing IL-13 in oxazolone-induced colitis (Baird *et al.* 1998; Heller *et al.* 2002). However, considering the heterogeneity of UC, many aspects of the mechanisms involving IL-4 and IL-13 and the receptors through which they signal remain undefined.

The importance of Th2 cytokines, in particular IL-13 in oxazolone colitis was first highlighted in a study by Heller and colleagues. IL-13 production by NK T cells was identified as the main inducer of disease in C57BL/10 mice (Heller *et al.* 2002). Subsequently, restimulation of colonic epithelial cells isolated from UC patients also demonstrated increased levels of IL-13 production compared with control patients (Heller *et al.* 2005; Fichtner-Feigl *et al.* 2006). In further studies, IL-13 was described as a key effector cytokine in UC acting on epithelial cell function and initiating apoptosis (Heller *et al.* 2005). The addition of IL-13 *in vitro* to HT-29 epithelial cell monolayers led to an increased expression of the pore-forming tight junction protein claudin-2. Increased expression of this protein is associated with increased epithelial barrier permeability. As a consequence, small antigens enter the gut and the loss of ions and water into the intestinal lumen leads to diarrhoea. These results suggest that changes in barrier function in human UC are not only due to NK T cell cytotoxicity on epithelial cells but also because of the subtle changes induced by IL-13 (Heller *et al.* 2005).

Furthermore, recent studies have shown IL-13<sup>-/-</sup> BALB/c mice to be protected from oxazolone colitis, confirming the important role of IL-13 in mediating colitis in BALB/c mice (Weigmann *et al.* 2008). Neutralizing IL-4 in SJL/J mice (Baird *et al.* 1998) or IL-4 deficiency in C57BL/6 mice (Iijima *et al.* 2004) also protects mice against the induction of colitis. Together with the important role of IL-13 in oxazolone, these data

suggest a major role for IL-4 receptor-alpha (IL-4R $\alpha$ ) in UC. However, there are no studies using IL-4R $\alpha$  gene-deficient mice in the oxazolone model. In this study, we adopted an oxazolone-induced model for UC in BALB/c and IL-4R $\alpha^{-/-}$  mice (Mohrs *et al.* 1999), as well as IL-4/IL-13 $^{-/-}$  mice in order to define a role for IL-4 and IL-13 signalling through IL-4R $\alpha$  in disease pathology.

Our data confirms previous studies showing the importance of IL-4 and IL-13 production in oxazolone-induced colitis. However we show for the first time that blocking IL-4 and IL-13 signalling via the IL-4R $\alpha$  using mice deficient for this receptor (IL-4R $\alpha^{-/-}$ ) does not protect mice from colitis. In fact IL-4R $\alpha^{-/-}$  mice develop exacerbated pathology with significantly higher mortality than oxazolone-treated BALB/c mice. These data suggest a regulatory role for IL-4R $\alpha$  in oxazolone colitis. We confirm that IL-13 mediates colitis in IL-4R $\alpha^{-/-}$  mice, as disrupting IL-13 but not IL-4 production in wild type and IL-4R $\alpha^{-/-}$  mice rescues them from the pathology associated with oxazolone colitis. This highlights a role for IL-4R $\alpha$ -independent signalling of IL-13 to induce colitis. Recently IL-13 has been shown to signal via the IL-13R $\alpha$ 2 by up-regulated expression of the AP-1 transcription factor. This makes IL-13R $\alpha$ 2 a likely candidate for IL-13-mediated oxazolone colitis. We show that IL-13R $\alpha$ 2 is expressed on many cell types in the spleen, peritoneal cavity and lymph node and that oxazolone down-regulates this expression in neutrophils. Lastly, soluble levels of IL-13R $\alpha$ 2 in serum were significantly reduced in mice susceptible to oxazolone colitis. Therefore we highlight the importance of IL-4R $\alpha$ -independent signalling of IL-13 in mediating oxazolone colitis and suggest a role for IL-13R $\alpha$ 2 in causing the pathology associated with disease.

## MATERIALS AND METHODS

### *Mice.*

Male BALB/c mice (6-8 weeks old) and IL-4<sup>-/-</sup> (Noben-Trauth *et al.* 1996), IL-13<sup>-/-</sup> (McKenzie *et al.* 1998), IL-4Rα<sup>-/-</sup> (Mohrs *et al.* 1999) and IL-13 transgenic (Emson *et al.* 1998) mice on a BALB/c background were used in oxazolone colitis experiments. Furthermore, IL-4<sup>-/-</sup>, IL-13<sup>-/-</sup> and IL-4Rα<sup>-/-</sup> mice were intercrossed to generate IL-4/IL-13, IL-4Rα/IL-4 and IL-4Rα/IL-13 double-deficient strains. All mice were housed in specific pathogen-free conditions at the University of Cape Town, South Africa and experiments were approved by the University's Animal Ethics Committee.

### *Induction of colitis by haptening agent oxazolone.*

Oxazolone-mediated colitis was induced in BALB/c mice by modifying previously described methods (Boirivant *et al.* 1998; Neurath *et al.* 2002; Kojima *et al.* 2004). Anesthetized mice were sensitized on the shaved abdomen by application of 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich) in 100% ethanol (15 µl) followed 7 days later by intrarectal administration of 1% oxazolone in 50% ethanol (150µl). Control mice were sensitized and challenged with ethanol only (controls were included for all mouse groups used, data not shown). For rectal administration, a metal catheter was lubricated and inserted 4cm into the lumen of the colon via the anus. After injection of the oxazolone/ethanol solution and removal of the catheter, mice were held vertically for 30sec to ensure distribution of the oxazolone within the entire colon. Mice were weighed and monitored daily and killed 3 days post-challenge for immunopathological analyses or 7 days post-challenge for survival studies.

### *Disease activity index (DAI).*

Oxazolone treated BALB/c mice develop rapid-onset colitis marked by weight loss and distress. To monitor disease progression, mice were weighed and monitored for distress and gastrointestinal symptoms 1-2 times daily depending on severity. Weight loss was measured as a percentage of starting weight and distress was scored at day 2 post-challenge according to appearance, clinical signs and natural or provoked behaviour (see Table 1 of Appendix A) (Wolfensohn and Lloyd 1998). Mice were scored on a scale of 0-16 and killed if reaching the most severe score (16) or losing ≥20% body weight. Survival studies were performed with 10 mice/group and the same criteria were monitored as mentioned above. Colon length was measured from the anus to the caecum and recorded as an indication of inflammation.

***Measurement of Myeloperoxidase (MPO) activity.***

MPO activity was analyzed as a marker for neutrophil infiltration in inflamed colon tissue. Colon tissue from individual mice was weighed, resuspended in HTAB buffer, homogenized and sonicated for 20 pulses at 9watts. After centrifugation for 10min at 10000rpm, the supernatants were collected and added to a 96 well maxisorp microtitre plate (Nunc, Reskilde, Denmark) in serial dilutions. A horseradish peroxidase (HRP) standard (Sigma, Steinheim, Germany) was added to the plate at a starting concentration of 0.25U with 3-fold serial dilutions to quantify MPO activity. O-dianisidine was added and after incubation for 10min at 35°C, H<sub>2</sub>O<sub>2</sub> was added to start the colour reaction. The reaction was stopped with 1% NaN<sub>3</sub> and read at 460nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany). Values are expressed as units per gram of tissue from individual mice (see Appendix A for additional reagents for all experiments).

***Histological assessment of colitis.***

Distal colon sections taken 1 cm from the anus were fixed in ice cold 4% phosphate-buffered formalin to prevent tissue autolysis. Paraffin embedded tissue was cut into sections and stained with haematoxylin and eosin (H&E) for inflammatory cells or Periodic acid-Schiff reagent (PAS) for mucus producing goblet cells. Semi-quantitative histopathological grading of oxazolone-induced colitis was adjusted from a previous study, (Iijima *et al.* 2004). Essentially, mice were graded on 5 criteria, 1) presence of mononuclear cells, 2) reduced goblet cells, 3) epithelial injury, 4) granulocyte infiltration and 5) oedema. Each criteria were scored from 0-3 and the total score was added resulting in a total additive score of between 0 (no colitis) and 15 (maximal colitis activity). Histology sections were processed and stained by the Department of Surgery, Groote Schuur Hospital.

***Isolation and restimulation of splenocytes.***

Individual or pooled spleens from oxazolone treated mice were collected in complete Iscoves Modified Dulbeccos Medium (IMDM) supplemented with; 10% FCS (Gibco Life Technologies, Paisly, UK), penicillin, streptomycin, 1mM sodium pyruvate, 50µM B2-ME (Sigma) and NEAA (Invitrogen). Single cell suspensions were obtained by pressing spleens through a 70µm cell strainer (Falcon, New Jersey, USA) and centrifugation at 1200rpm for 5min at 4°C in a 15ml tube. The pelleted cells were depleted of RBCs by hypotonic lysis in Red Cell Lysis Buffer and the centrifugation step

was repeated. Cells were resuspended in 2ml complete IMDM and stained with Trypan Blue (Sigma-Aldrich, Irvine, UK) to count viable cells in a haemocytometer. Splenocytes were diluted to  $1 \times 10^6$  cells/ml and cultured in a 96-well plate in complete IMDM with anti-CD3 (clone: 145-2C11,  $10 \mu\text{g/ml}$ ) for 48h at  $37^\circ\text{C}$  and 5%  $\text{CO}_2$ . Supernatants were collected and stored at  $-80^\circ\text{C}$ .

### ***Cytokine and IL-13R $\alpha$ 2 ELISAs.***

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants and soluble IL-13R $\alpha$ 2 levels in serum. Maxisorp microtitre plates (Nunc, Reskilde, Denmark) were coated with purified anti-IL-4 (clone: 11B11,  $2 \mu\text{g/ml}$ ), anti-IFN- $\gamma$  (clone: An18KL6,  $1 \mu\text{g/ml}$ ), anti-IL-13 (clone: 38213,  $1 \mu\text{g/ml}$ , BD Pharmingen, San Diego, USA) or anti-IL-13R $\alpha$ 2 (goat anti-mouse, R&D systems, U.S.A.), diluted in phosphate buffered saline (PBS, pH 9.5) and incubated overnight at  $4^\circ\text{C}$ . Plates were blocked for 3h at  $37^\circ\text{C}$  and serially diluted standards, purified recombinant IL-4, IFN- $\gamma$  or IL-13 (BD Pharmingen) and IL-13R $\alpha$ 2 (R&D systems) were added. Cell supernatants or serum was added, diluted in ELISA dilution buffer and incubated overnight at  $4^\circ\text{C}$ . Biotinylated anti-mouse secondary antibodies for IL-4 (clone: BVD6-24G2), IFN- $\gamma$  (clone: XMG1.2) or IL-13 (goat anti-mouse,  $0.5 \mu\text{g/ml}$ , BD Pharmingen) and IL-13R $\alpha$ 2 (goat anti-mouse,  $0.5 \mu\text{g/ml}$ , R&D systems) were added for 3h at  $37^\circ\text{C}$  followed by HRP labelled streptavidin. Subsequently, plates were incubated with TMB Peroxidase Substrate (KPL, USA), the colour reaction stopped with  $2\text{M H}_2\text{SO}_4$  and absorption measured at 450nm. ELISA wash buffer was used to wash plates 4x between each step.

### ***Total IgE detection by ELISA.***

Total IgE levels were measured in the serum of oxazolone-treated mice (day 3 post-challenge) by sandwich ELISA (as above). Anti-mouse IgE (clone: 84.1C) mAb was used for coating, purified recombinant mouse IgE (BD Pharmingen) as a standard and alkaline phosphatase-labelled anti-mouse IgE mAb (23G3, Southern Biotechnology, USA) for detection. 4-Nitrophenylphosphate (PNP, Fluka, Switzerland) dissolved at  $1\text{mg/ml}$  in substrate buffer was added to all samples and the absorbance measured at 405nm.

### ***Flow cytometry.***

Lymphocytes and splenocytes were isolated as described above and resuspended in FACS buffer. Peritoneal cells were isolated by peritoneal lavage using 8ml of IMDM

supplemented with 10% FCS and resuspended in FACS buffer for staining. Natural killer cells (DX5<sup>+</sup>/CD3<sup>-</sup>), T cells (DX5<sup>-</sup>/CD3<sup>+</sup>) and NK T cells (DX5<sup>+</sup>/CD3<sup>+</sup>) were quantified in the spleen using biotinylated mAbs for pan natural killer cell (CD49b) (clone: DX5, BD Pharmingen) and fluorescein isothiocyanate (FITC) anti-CD3 (clone: 145-2C11) mAbs. IL-13 production by NK T cells was measured by intracellular staining with phycoerythrin (PE) labelled anti-IL-13 antibody (clone: eBio13A, eBioscience). Spleen cells,  $2 \times 10^5$  cells/well were stimulated with PMA (0.1  $\mu$ g/ml) and ionomycin (2  $\mu$ g/ml, both Sigma) supplemented with or without monensin (2  $\mu$ M, eBioscience) as a control, for 4h at 37°C. Intracellular expression of the decoy IL-13 receptor was measured with biotinylated anti-IL-13R $\alpha$ 2 (goat anti-mouse, R&D Systems) on various subpopulations of splenocytes, lymphocytes and peritoneal cells. Splenocytes were identified with anti-CD19 (clone: 1D3), anti-CD3 (clone: 145-2C11), anti-CD4 (clone: GK1.5) or anti-DX5/anti-CD3 and anti-CD11b/anti-GR-1 (clones: M1/70 and RB68C5) mAbs. Lymphocytes were identified with anti-CD19 or anti-CD4 mAbs and peritoneal cells were identified with anti-F4/80/anti-CD11b (clone: A3-1, Serotec, Oxford UK) or anti-CD11b/anti-GR-1 mAbs. Biotin-labelled antibodies were detected by streptavidin-allophycocyanin (APC). Antibodies were from BD Pharmingen (San Diego, CA) except where noted otherwise. Anti-Fc $\gamma$ RII/III antibody (clone: 2.4G2) was used in order to block non-antigen-specific binding of immunoglobulins to the Fc $\gamma$ II and Fc $\gamma$ III receptors. For intracellular staining, cells were stained according to the manufacturers instructions (eBioscience). Essentially, cells were stained for surface markers, fixed for 30min with fixation/permeabilization solution, permeabilized for 15min and stained with anti-IL-13 (PE) or anti-IL-13R $\alpha$ 2 (Bio) antibodies. Cells were acquired using a FACSCalibur (Beckton-Dickinson, Ferndale, South Africa) and data were analysed with Cellquest (BD biosciences) or Flowjo software (Treestar). Histograms were represented as “percentage of maximum” which normalizes mean fluorescence per histogram based on the number of gated cells.

### ***Statistical analysis.***

Values are given as mean  $\pm$  SEM, and significant differences were determined using unpaired two-tailed students *t* test or one-way ANOVA using a Bonferroni post-test (GraphPad Prism). Values of *p* < 0.05 were considered significant.

## RESULTS

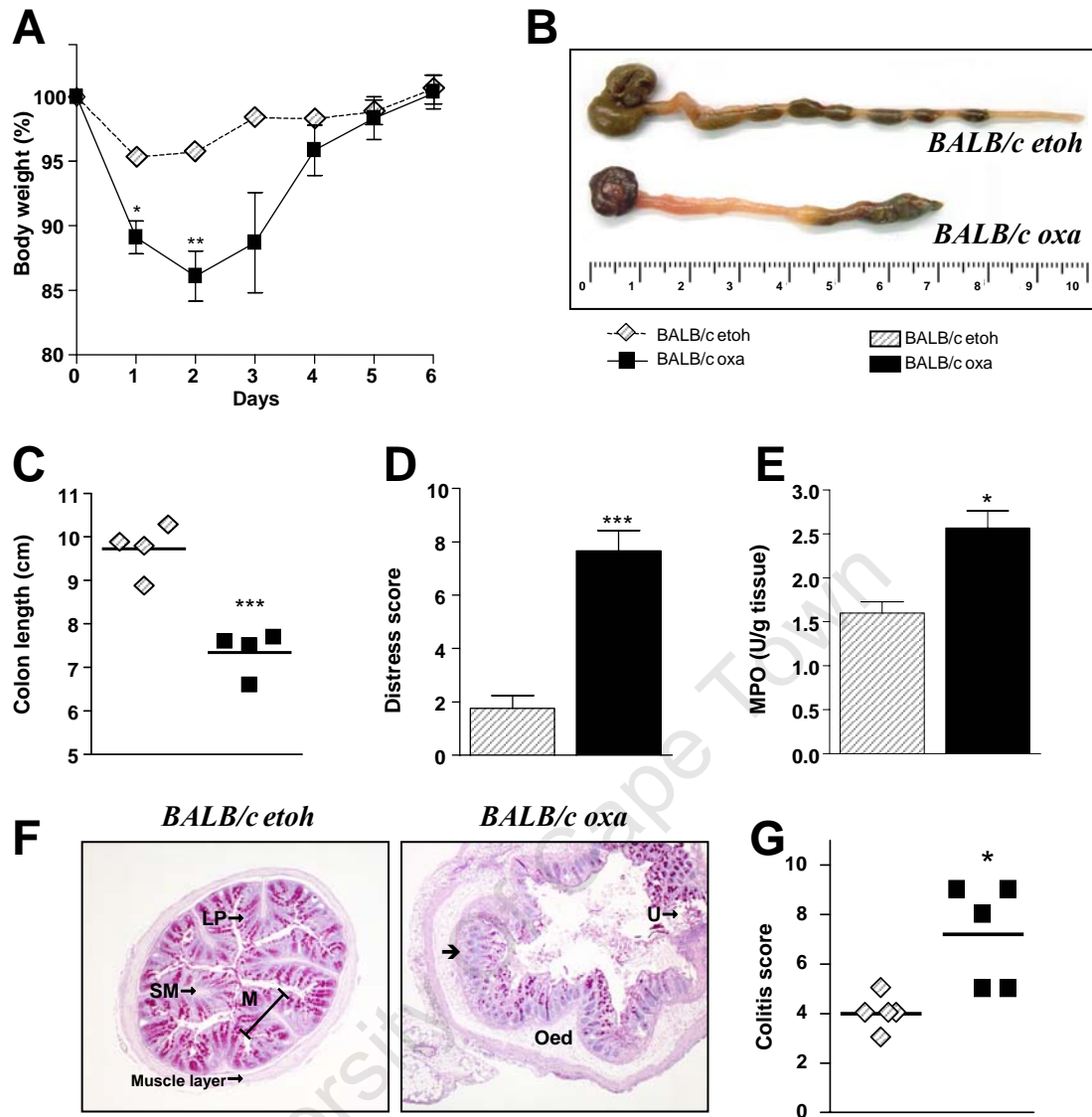
### ***Oxazolone induces an UC-like Th2-mediated inflammation in BALB/c mice.***

We presensitized male BALB/c mice with 3% oxazolone 7 days before intrarectal challenge with 1% oxazolone solution. As shown in Figure 1A, oxazolone induced a rapid onset colitis marked by weight loss peaking by day 2 and leading to death of  $\geq 50\%$  mice, with surviving BALB/c mice recovering by day 6. Macroscopic examination 3 days post-challenge showed severe colitis limited to the distal half of the colon (Figure 1B) with inflammation associated colon shortening compared to ethanol-only control BALB/c mice (Figure 1C).

BALB/c mice treated with oxazolone showed significant signs of distress compared to control mice (Figure 1D). Distress was scored according to; appearance, clinical signs, natural and provoked behaviour. The colonic MPO activity was measured at day 3 and indicated significantly increased levels of neutrophil infiltration compared to ethanol control group (Figure 1E). Microscopic examination of the distal colon showed superficial inflammation characterized by epithelial cell loss, occasional patchy ulceration and pronounced depletion of mucous producing-goblet cells (Figure 1F). Furthermore, the lamina propria presented with a mixed inflammatory cell infiltrate and the submucosal layer had oedema with a few inflammatory cells present. The outer muscle layer showed little evidence of inflammation. Oxazolone-induced colitis in BALB/c mice was scored according to; presence of mononuclear cells, oedema, infiltration of granulocytes, epithelial layer disruption and loss of mucus production (Figure 1G). The histopathological picture of oxazolone-induced colitis highly resembles that seen in human UC. As 50% ethanol alone has also been shown to induce a mild colitis in the colon, mice treated with ethanol-only were included as controls for all experiments (Andrade *et al.* 2003).

To characterize the systemic immune response in oxazolone colitis, cytokine production by spleen cells stimulated with anti-CD3 antibodies was determined 3 days after final challenge. IL-4 and IL-13 were increased, while no significant difference was seen in IFN- $\gamma$  production compared with ethanol-only control mice (Figure 2A). Furthermore, increased levels of total IgE was detected in the serum of oxazolone-treated mice (Figure 2B). These data show that oxazolone colitis is associated with a polarized Th2 response in BALB/c mice.





**Figure 1: Oxazolone induces an UC-like colonic inflammation in male BALB/c mice.**

Oxazolone-colitis was induced in BALB/c mice by pre-sensitization with 3% oxazolone (oxa) in 100% ethanol before intrarectal challenge with 1% oxa in 50% ethanol (BALB/c oxa). Control mice were administered ethanol only (BALB/c etoh).

(A) Oxazolone induces rapid onset of wasting disease measured by body weight as a percentage starting weight (day 6 post-challenge).

(B) Macroscopic pictures show inflammation limited to the distal half of the colon.

(C) Inflammation is also shown by colon shortening in oxazolone-treated mice.

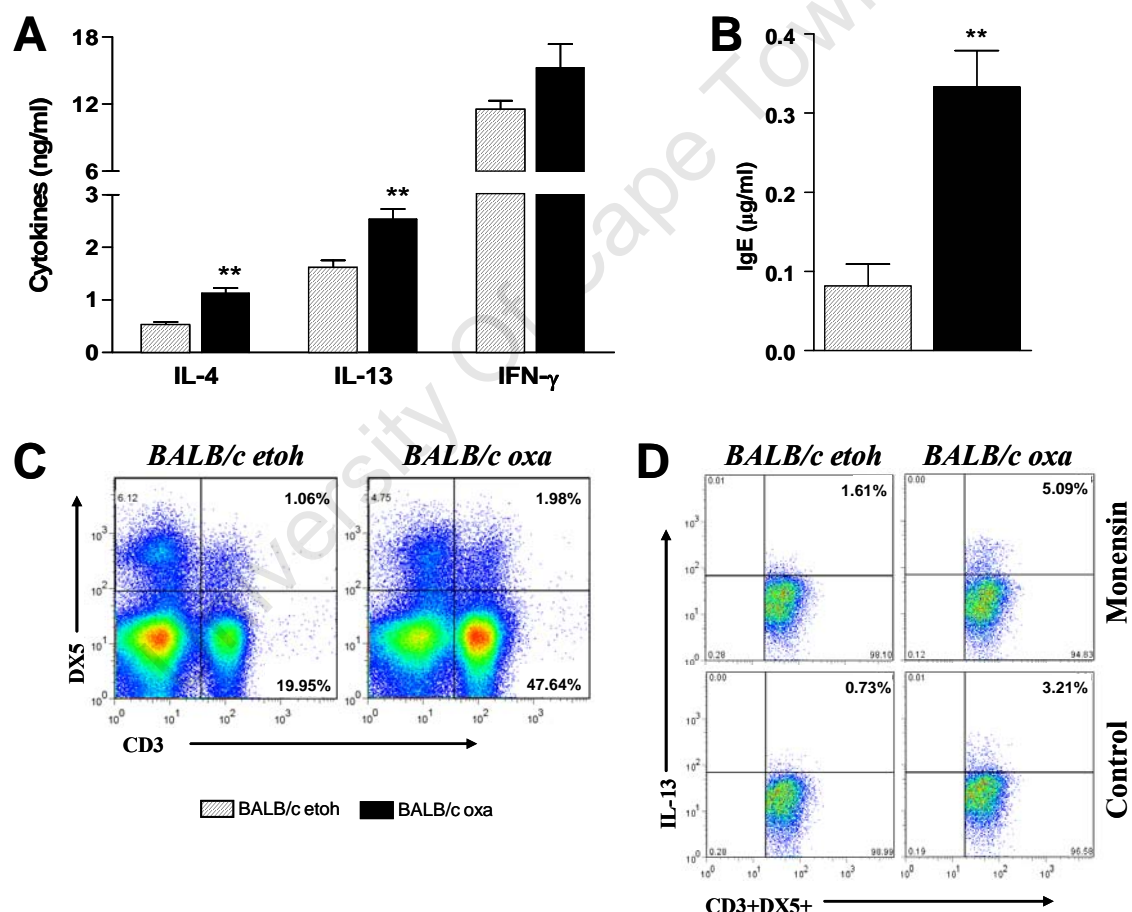
(D) Mice were monitored for distress and scored according to appearance, clinical signs, natural and provoked behaviour (day 2 post-challenge).

(E) Colonic myeloperoxidase (MPO) activity indicates neutrophil infiltration which was significantly increased in oxazolone-treated mice compared with control mice.

(F) Histological sections from BALB/c mice treated with oxazolone or ethanol-only controls were stained with PAS and representative photomicrographs (x40) are shown per group. SM =submucosa, M =mucosa, LP =lamina propria, → infiltration of mononuclear cells, oed =oedema and U =ulceration.

(G) Semi-quantitative histopathological assessment of colitis activity was performed based on the presence of mononuclear cells, oedema, epithelial damage, loss of goblet cells and granulocyte infiltration. All data represents mice killed 3 days post-challenge unless indicated. Data represents >3 individual experiments (n=4-10 mice) with mean values ± SEM and \* = p <0.05, \*\* = p <0.01 and \*\*\* = p <0.001 vs. BALB/c etoh-only control mice.

As previous studies have shown NK T cells, which secrete IL-13, to mediate the pathology in oxazolone colitis we measured the percentage of these cells in BALB/c mice (Figure 2C). We identified and quantified NK T cells in pooled spleens of oxazolone-treated mice by FACS analysis of surface markers for both NK and T cells. Representative dot plots showed a 2-fold increase in percentage NK T cells ( $DX5^+/CD3^+$ ) in oxazolone-treated mice compared with controls (Figure 2C). Using intracellular FACS analysis of  $DX5^+/CD3^+$  NK T cells, we show an increase in IL-13 production by these cells in oxazolone-treated BALB/c mice compared to ethanol-only controls (Figure 2D). Together, these data demonstrate that colonic inflammation characteristic of oxazolone colitis in BALB/c mice is associated with a polarized Th2 response.



**Figure 2: Oxazolone induces a Th2 response in BALB/c mice**

IL-13 producing NK T cells have been shown to cause the pathology associated with oxazolone-induced colitis. Systemic T lymphocyte responses were assessed from spleen cells of oxazolone treated mice.

**(A)** Triplicate cultures of pooled spleen cells stimulated with anti-CD3 antibody were used to measure *ex vivo* IL-4, IL-13 and IFN- $\gamma$  cytokine production by ELISA.

**(B)** Serum was collected from mice at day 3 post-challenge to determine total IgE levels by ELISA.

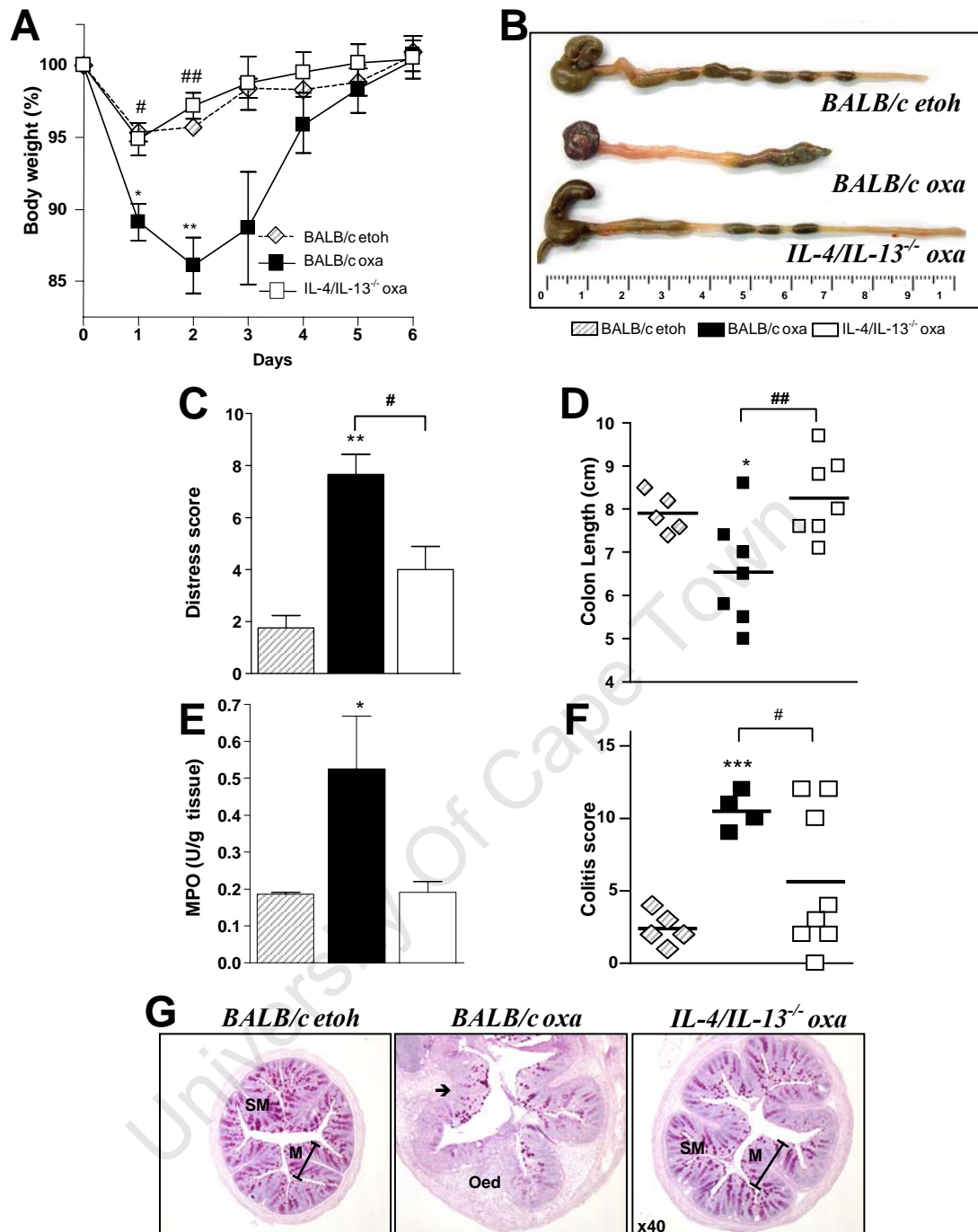
**(C)** Pooled spleen cells were isolated from oxazolone treated and control mice and stained with a pan-NK cell marker (DX5) and CD3 for T cells. Representative dot plots show percentage NK T and CD3 cells.

**(D)** Spleen cells were stimulated with PMA and ionomycin with or without Monensin (golgi blocker) and stained for DX5, CD3 and IL-13, to show IL-13 production by NK T cells. Data represents 3 individual experiments (n=4-10), mean  $\pm$  SEM and \*\* =  $p < 0.01$  vs. BALB/c etoh control.

***Mice deficient in IL-13 are protected from oxazolone-induced colitis.***

The importance of IL-4 and IL-13 cytokines in oxazolone colitis has been shown by previous studies in SJL/J and C57BL/10. To determine whether IL-4 and IL-13 play such a crucial role in BALB/c mice, we treated mice deficient for both IL-4 and IL-13 (IL-4/IL-13<sup>-/-</sup>) with oxazolone and compared the disease outcome to wild type controls. As shown in Figure 3A, IL-4/IL-13<sup>-/-</sup> mice were protected from weight loss as they rapidly regained their initial body weight and showed no macroscopic signs of inflammation (Figure 3B), with the colon length remaining significantly longer than oxazolone-treated BALB/c mice (Figure 3D). Distress was scored as mentioned above and MPO activity was measured 3 days after challenge with oxazolone and showed IL-4/IL-13<sup>-/-</sup> mice to have symptoms indistinguishable from ethanol control mice (Figure 3C and 3E). In addition, IL-4/IL-13<sup>-/-</sup> mice presented with significantly less histopathology when compared with oxazolone treated BALB/c mice (Figure 3F and 3G). As expected, *in vitro* stimulated splenocytes from IL-4/IL-13<sup>-/-</sup> mice did not produce IL-4 or IL-13 (Figure 4A). Furthermore, numbers of T cells and NK T cells were reduced (Figure 4B).

These data confirms the importance of IL-4 and/or IL-13 signalling in mediating oxazolone-induced colitis in BALB/c mice. To determine the individual roles of IL-4 and IL-13 we treated both IL-4<sup>-/-</sup> and IL-13<sup>-/-</sup> BALB/c mice with oxazolone and compared the disease onset with wild type mice. Table 1 and Table 2 summarize our data, which suggests that mice deficient in IL-4 production were not completely protected from oxazolone-induced colitis. This was shown by a significant increase in weight loss, distress, colitis and IL-13 production compared with BALB/c ethanol-only control mice.



**Figure 3: IL-4/IL-13 mediates oxazolone-induced colitis in BALB/c mice.**

Mice deficient in both IL-4 and IL-13 (IL-4/IL-13<sup>-/-</sup>) were protected from the rapid onset of colitis induced by oxazolone shown by;

(A) Reduced loss of body weight as percentage starting weight compared with BALB/c oxa mice (day 6).

(B) Normal colon appearance in macroscopic pictures.

(C) Significantly less distress, according to severity of symptoms with a maximum score of 16 (day 2).

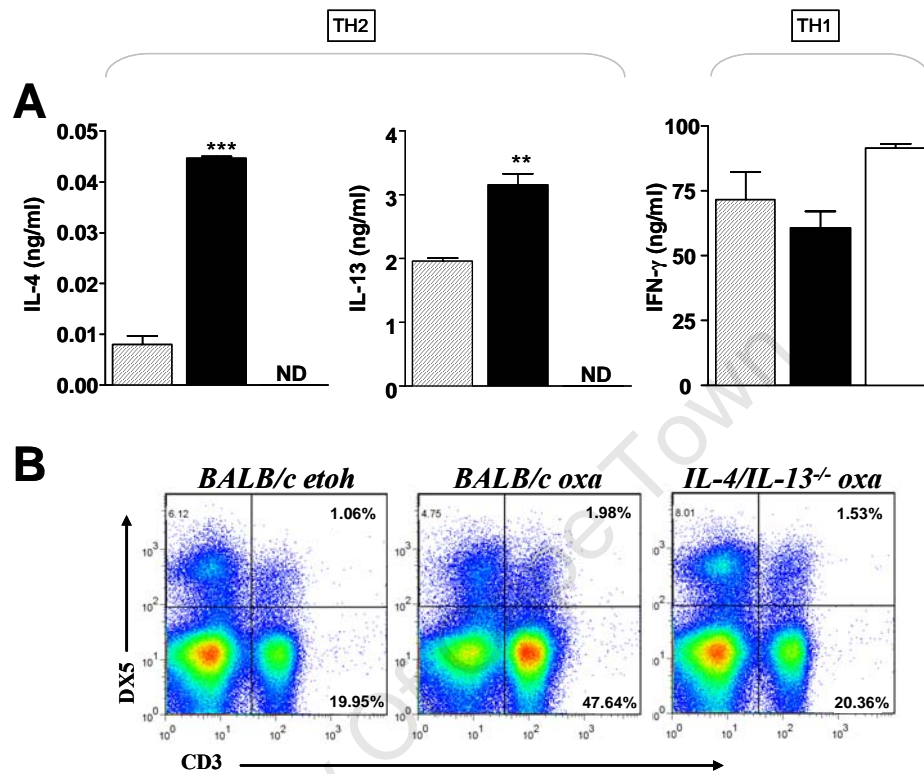
(D) Normal colon length, indicating reduced inflammation.

(E) Reduced colonic MPO activity comparable to control mice.

(F) Significantly reduced colitis score based on a semi-quantitative histopathological assessment.

(G) Reduced or no oxazolone-induced inflammation shown in PAS-stained sections of the distal colon, while BALB/c mice show oedema (oed), reduced mucous production and inflammatory infiltrates (→).

SM = submucosa and M = mucosa. All data represents mice killed 3 days post-challenge unless indicated. Data represents 3 individual experiments (n=6-8 mice) with mean values ± SEM and \* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs. BALB/c etoh controls and # = p < 0.05 and ## = p < 0.01 BALB/c oxa vs. IL-4/IL-13<sup>-/-</sup> oxa.



**Figure 4: Abrogated Th2 response to oxazolone in IL-4/IL-13 deficient mice.**

Systemic T lymphocyte responses were assessed from spleen cells of oxazolone treated IL-4/IL-13<sup>-/-</sup> mice and compared to oxazolone treated or control wild type BALB/c mice.

**(A)** Systemic cytokine production from splenocytes were analysed by ELISA showing expected abrogation of IL-4 and IL-13 and no effect on IFN-γ levels. This confirms the functional deletion of IL-4/IL-13.

**(B)** Pooled spleen cells isolated from oxazolone treated and control mice were stained with a pan-NK cell marker (DX5) and CD3 for T cells.

Data represents 2 individual experiments (n = 6-8), with mean ± SEM and \*\* = p < 0.01 and \*\*\* = p < 0.001 vs. BALB/c etoh control.

In contrast, mice deficient in IL-13 production were significantly protected from oxazolone-induced colitis. This was shown by a significant decrease in weight loss, distress and colitis with abrogated IL-13 production compared to oxazolone-treated BALB/c mice (Table 1 and Table 2). Furthermore, we showed that mice over expressing IL-13 (IL-13 trans) presented with a more severe oxazolone colitis than BALB/c mice. Data represented in Table 1 and Table 2 show significantly increased weight loss, distress and colitis with high levels of IL-13 production in IL-13 trans compared with BALB/c oxazolone-treated mice. These data confirm a crucial role for IL-13 in UC as recently shown by (Weigmann *et al.* 2008).

**Table 1: Disease activity in oxazolone treated gene-deficient or transgenic BALB/c mice.**

Groups	Body weight <sup>a</sup>	Survival <sup>b</sup>	Distress Score <sup>c</sup>	Colon length (cm)
BALB/c etoh	98.19 ± 1.6	83.33 ± 15.2	3.33 ± 0.2	8.23 ± 0.5
BALB/c oxa	91.66 ± 1.8 *	57.14 ± 18.7 *	9.38 ± 0.9 ***	6.81 ± 0.4 *
IL-4 <sup>-/-</sup>	89.85 ± 1.4 **	(62.50 ± 17.1)	9.34 ± 1.2 **	7.84 ± 0.6
IL-13 <sup>-/-</sup>	98.74 ± 1.5 #	(87.50 ± 11.7)	5.50 ± 0.5 #	9.53 ± 0.6
IL-13 trans	88.50 ± 1.2 #	-----	12.33 ± 1.2 ##	6.5 ± 0.6 **
IL-4Rα/IL-4 <sup>-/-</sup>	87.21 ± 2.2 **	27.27 ± 13.4 *	9.00 ± 1.4 *	7.47 ± 0.1 *

<sup>a</sup> Body weight is shown as percentage starting weight 2 days post-challenge.

<sup>b</sup> Percentage survival shown for 7 days post-challenge. Brackets are from 3 day survival curves only.

<sup>c</sup> Distress is scored according to appearance, clinical signs, natural and provoked behaviour.

\* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs BALB/c etoh control.

# = p < 0.05 and ## = p < 0.01 BALB/c oxa vs. genetically modified mice.

**Table 2: Inflammatory response to oxazolone in gene-deficient or transgenic BALB/c mice.**

Groups	Colitis score <sup>a</sup>	IL-4 (ng/ml)	IL-13 (ng/ml)	IFN-γ (ng/ml)
BALB/c etoh	2.78 ± 0.5	0.013 ± 0.005	0.24 ± 0.004	11.26 ± 0.2
BALB/c oxa	8.33 ± 0.8 ***	0.076 ± 0.01 **	0.88 ± 0.01 ***	43.08 ± 8.3 *
IL-4 <sup>-/-</sup>	6.78 ± 1.3 *	ND	0.51 ± 0.004 **	29.60 ± 8.2
IL-13 <sup>-/-</sup>	5.50 ± 0.6 #	0.012 ± 0.06 ##	ND	68.44 ± 14.1
IL-13 trans	12.00 ± 0.5 ##	0.063 ± 0.005	3.24 ± 0.04 ###	43.28 ± 1.5 *
IL-4Rα/IL-4 <sup>-/-</sup>	9.57 ± 0.5 ***	ND	0.28 ± 0.004 ###	34.10 ± 0.2 *

<sup>a</sup> Semi-quantitative histopathological assessment of colitis activity was performed based on the presence of mononuclear cells, oedema, epithelial damage, loss of goblet cells and granulocyte infiltration.

\* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs BALB/c etoh control.

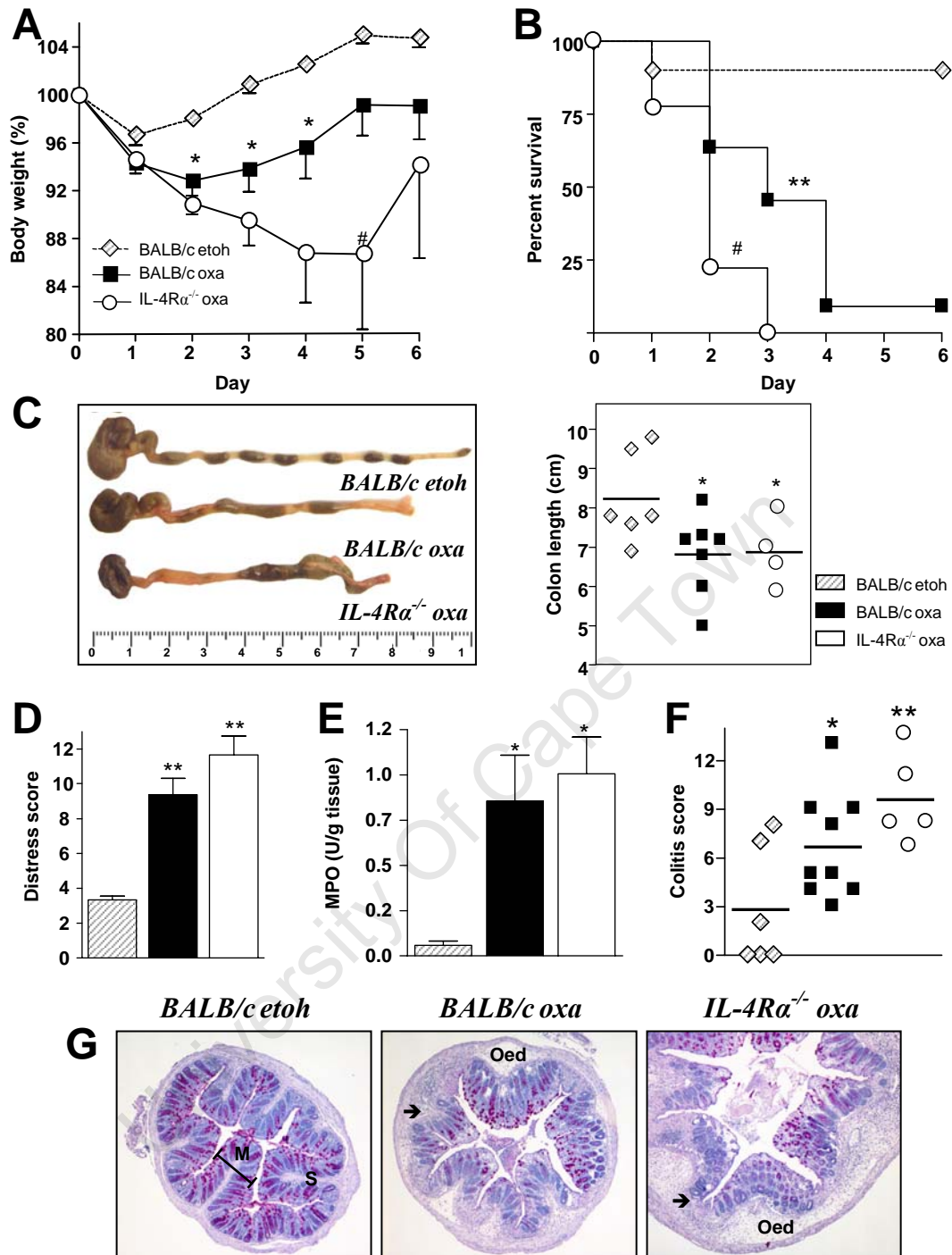
## = p < 0.01 and ### = p > 0.001 BALB/c oxa vs. genetically modified mice.

***Deletion of the common receptor for IL-4 and IL-13, IL-4R $\alpha$  exacerbates oxazolone-induced colitis.***

IL-4 and IL-13 both signal via the IL-4R $\alpha$ . Considering our previous results (supported by Weigmann *et. al.* 2008) which demonstrate the importance of IL-13 in mediating oxazolone-colitis, we expected to inhibit disease development in BALB/c mice lacking this receptor on all cells (IL-4R $\alpha$ <sup>-/-</sup> mice). Interestingly, IL-4R $\alpha$ -deficient mice developed more severe oxazolone colitis than BALB/c mice. As shown in Figure 5A, IL-4R $\alpha$ <sup>-/-</sup> mice weighed significantly less than both oxazolone or ethanol-treated BALB/c mice, with a mortality rate > 25% (Figure 5B). In some experiments oxazolone-treatment even resulted in total loss of all IL-4R $\alpha$ <sup>-/-</sup> mice, either due to death or killing of mice that had lost  $\geq 20\%$  body weight. Macroscopic examination 3 days post-challenge showed severe colitis limited to the distal half of the colon with inflammation associated colon shortening compared with oxazolone-treated BALB/c mice (Figure 5C). IL-4R $\alpha$ <sup>-/-</sup> mice also showed significant signs of distress (Figure 5D) and MPO levels comparable to BALB/c mice (Figure 5E). Microscopic examination of the distal colon showed superficial inflammation characterized by epithelial cell loss, patchy ulceration and pronounced depletion of mucous producing-goblet cells. The oxazolone-induced colitis, scored according to; presence of mononuclear cells, oedema, infiltration of granulocytes, epithelial layer disruption and loss of mucus production was significantly increased in IL-4R $\alpha$ <sup>-/-</sup> mice compared to oxazolone-treated BALB/c mice (Figure 5F and 5G).

IL-4R $\alpha$ <sup>-/-</sup> mice have an impaired systemic Th2 response in oxazolone colitis with decreased IL-4, IL-13 and no total IgE production (Figure 6A and 6B). Interestingly, these mice develop severe colitis despite impaired systemic IL-13 production. The percentage of NK T and CD3<sup>+</sup> cells remains elevated, comparable to oxazolone-treated BALB/c mice (Figure 6C). These data demonstrate that mice deficient in the common receptor for IL-4 and IL-13 signalling, IL-4R $\alpha$  develop severe oxazolone-induced colitis. This result was unexpected as it has been shown previously that both ligands of IL-4R $\alpha$  play an essential role in oxazolone colitis.



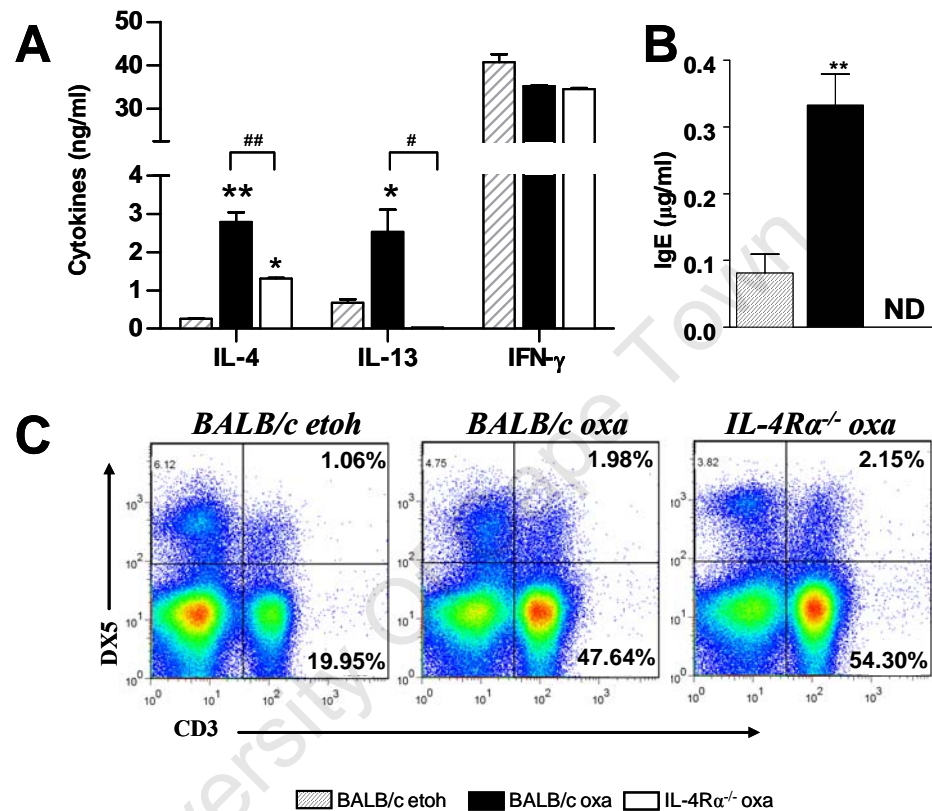


**Figure 5: Deletion of IL-4Rα exacerbates oxazolone-induced colitis.**

Both IL-4 and IL-13 signal via the IL-4Rα and deleting this receptor is expected to protect mice from oxazolone-induced colitis. However, IL-4Rα<sup>-/-</sup> mice show severe disease onset compared to BALB/c mice in response to oxazolone. This is shown by:

(A) Significantly increased weight loss peaking at 5 days post-challenge (day 6). (B) Higher mortality with complete loss of mice in some experiments (day 6). (C) Significantly more distress, scored according to severity of symptoms (day 2). (D) Severe macroscopic colon inflammation with reduced colon length. (E) MPO activity comparable to oxazolone treated BALB/c mice. (F) Increased colitis score based on a semi-quantitative histopathological assessment. (G) Severe inflammation shown in PAS-stained sections of the distal colon, with oedema (oed), reduced mucous production and inflammatory infiltrates (→). All data represents mice killed 3 days post-challenge unless indicated. Data represents 3 individual experiments (n=4-10 mice) with mean values ± SEM and \* = p < 0.05 and \*\* = p < 0.01 vs. BALB/c etoh control and # = p < 0.05 BALB/c oxa vs. IL-4Rα<sup>-/-</sup> oxa.





**Figure 6: IL-4Rα-deficient mice have exacerbated colitis despite an impaired Th2 response.**

Systemic T lymphocyte responses were assessed from spleen cells of oxazolone treated IL-4Rα<sup>-/-</sup> mice and compared to oxazolone treated or control wild type BALB/c mice.

(A) Despite the severity of disease onset, systemic cytokine production from the spleen analyzed by ELISA showed significantly reduced levels of IL-13 production.

(B) Serum IgE levels were measured by ELISA.

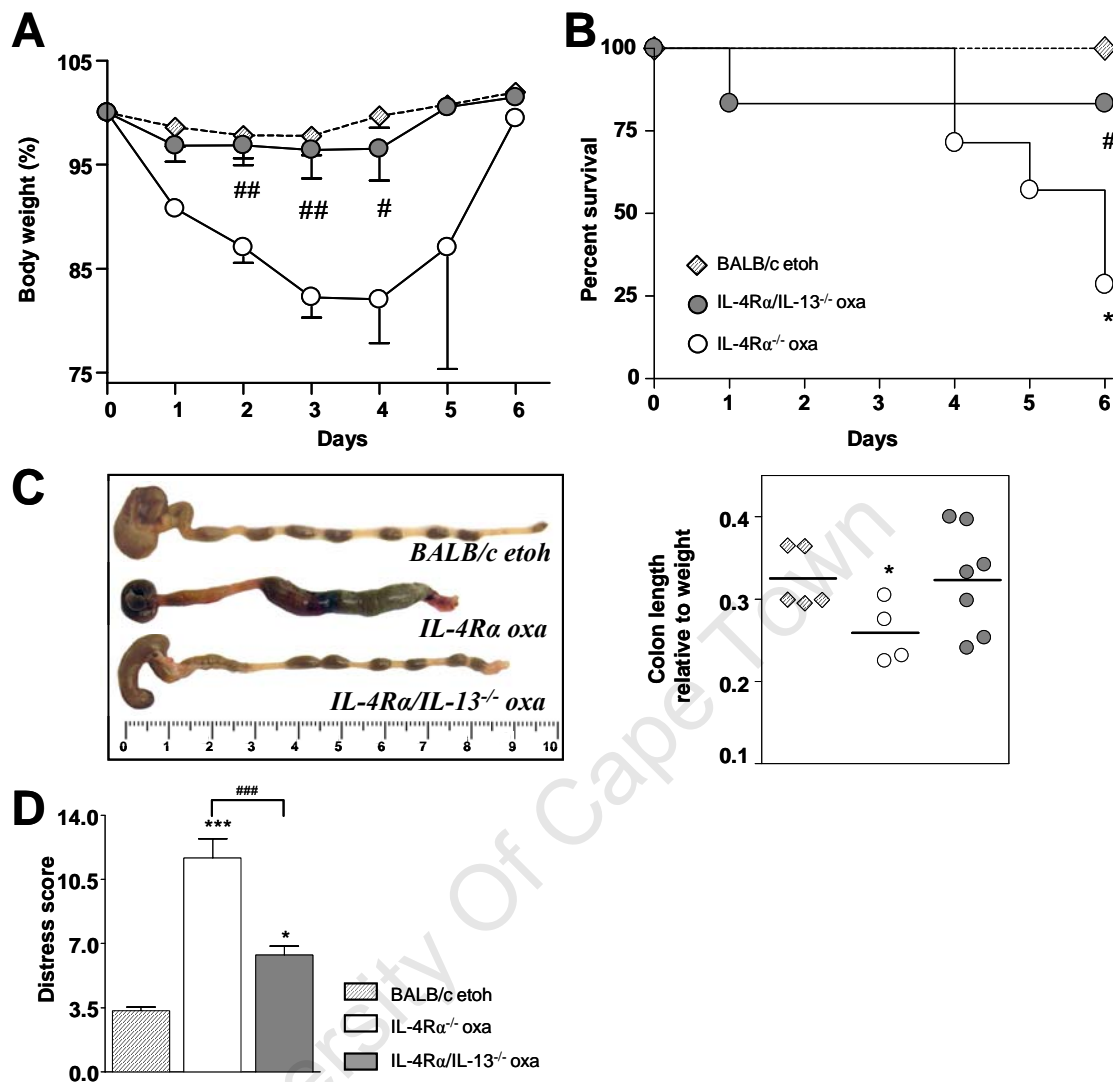
(C) Pooled spleen cells isolated from oxazolone treated and control mice were stained with a pan-NK cell marker (DX5) and CD3 for T cells. Exacerbated disease in IL-4Rα<sup>-/-</sup> mice corresponded with increased numbers of NK T cells. Data represents 2 individual experiments (n=4-10), with mean ± SEM and \* = p < 0.05 \*\* = p < 0.01 vs. BALB/c etoh control and # = p < 0.05 ## = p < 0.01 and ### = p < 0.001 BALB/c oxa vs. IL-4Rα<sup>-/-</sup> mice.

***IL-13 causes colitis in IL-4R $\alpha$ -deficient mice.***

After confirming the importance of IL-13 we wanted to determine if IL-13 causes oxazolone-induced colitis in IL-4R $\alpha$ -deficient mice, possibly by signalling through another receptor. IL-4R $\alpha$ <sup>-/-</sup> mice were crossed with IL-13<sup>-/-</sup> mice generating IL-4R $\alpha$ /IL-13-double deficient BALB/c mice. Our data showed the protection of IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice from oxazolone-induced colitis with weight loss comparable to ethanol-only control mice (Figure 7A). Furthermore, IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice had a lower mortality rate (Figure 7B) and macroscopic colon appearance similar to ethanol-only control mice (Figure 7C). As IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice were smaller at 6-8 weeks old, colon length was measured relevant to starting weight before oxazolone treatment and was comparable to ethanol-only control BALB/c mice (Figure 7C). Although IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice were distressed compared to ethanol-only controls, they were significantly less distressed than oxazolone-treated IL-4R $\alpha$ <sup>-/-</sup> mice (Figure 7D).

Systemic T lymphocyte responses from stimulated spleen cells showed IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice maintaining high levels of IL-4 production comparable to oxazolone-treated IL-4R $\alpha$ <sup>-/-</sup> mice and no detectable levels of IL-13, confirming the efficiency of IL-13 deletion (Figure 8A). IFN- $\gamma$  production was not significantly different to ethanol-only control mice. Microscopic examination of the distal colon showed no significant superficial inflammation, no ulceration and very little depletion of mucous producing-goblet cells in IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice (Figure 8B). Furthermore, the lamina propria presented with very mild inflammatory cell infiltrate and only slight oedema of the submucosa in some mice. Scoring of colitis showed no significant difference between IL-4R $\alpha$ /IL-13<sup>-/-</sup> and ethanol control mice but significantly reduced colitis compared with oxazolone-treated IL-4R $\alpha$ <sup>-/-</sup> mice (Figure 8C). Lastly, neutrophil activity was significantly reduced in protected IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice compared with oxazolone-treated IL-4R $\alpha$ <sup>-/-</sup> mice (Figure 8D). The protection seen was specific to IL-13 as mice deficient in both IL-4R $\alpha$  and IL-4 (IL-4R $\alpha$ /IL-4<sup>-/-</sup>) were not protected from oxazolone colitis (data shown in Table 1 and 2). IL-4R $\alpha$ /IL-4<sup>-/-</sup> mice showed weight loss, mortality, distress and colon inflammation and shortening comparable to wild type BALB/c mice treated with oxazolone. Furthermore, these mice had no detectable IL-4 with significantly reduced levels of IL-13 production.

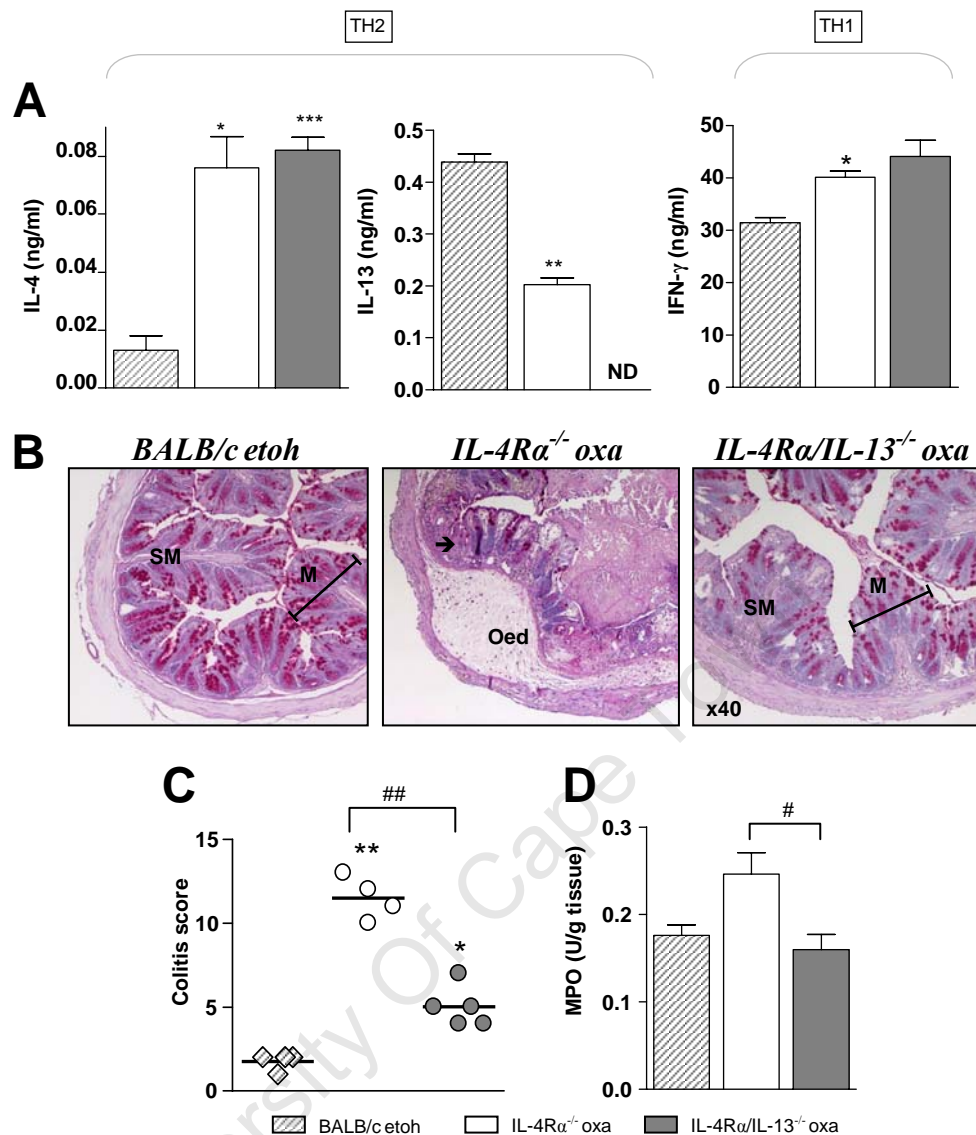
Together these data confirm that IL-13 is responsible for mediating pathology in oxazolone colitis and suggests that IL-13 signals independently of the IL-4R $\alpha$  to induce disease.



**Figure 7: IL-13 causes colitis in IL-4R $\alpha$ -deficient mice.**

To determine if IL-13 signalling independently of the IL-4R $\alpha$  would cause oxazolone-induced colitis, mice deficient in both the IL-4R $\alpha$  and IL-13 (IL-4R $\alpha$ /IL-13 $^{-/-}$ ) were treated with oxazolone. These mice were significantly protected from disease onset compared with IL-4R $\alpha^{-/-}$  oxazolone treated mice. The double knockout mice showed;

(A) Reduced weight loss comparable to ethanol-only control BALB/c mice (day 6), (B) decreased mortality (day 6), (C) normal colon appearance in macroscopic pictures and normal length relative to starting weight, and (D) significantly less distress (day 2). All data represents mice killed 3 days post-challenge unless indicated. Data represents 2 individual experiments (n=4-8) with mean values  $\pm$  SEM and \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs. BALB/c etoh control and # =  $p < 0.05$ , IL-4R $\alpha^{-/-}$  oxa vs. IL-4R $\alpha$ /IL-13 $^{-/-}$  oxa mice.



**Figure 8: IL-13 deficiency protects IL-4Rα<sup>-/-</sup> mice from colitis.**

Systemic T lymphocyte responses were assessed from spleen cells of oxazolone treated IL-4Rα/IL-13<sup>-/-</sup> mice and compared to oxazolone treated IL-4Rα<sup>-/-</sup> mice.

(A) IL-4Rα/IL-13<sup>-/-</sup> mice were protected from disease corresponding to abrogated levels of IL-13. Systemic cytokine production from the spleen was analysed by ELISA.

(B) Histological sections were stained with PAS and representative photomicrographs (x40) shown. SM = submucosa, M = mucosa, → = presence of mononuclear cells and oed = oedema.

(C) Semi-quantitative histopathological assessment of colitis activity was performed based on the presence of mononuclear cells, oedema, epithelial damage, loss of goblet cells and granulocyte infiltration.

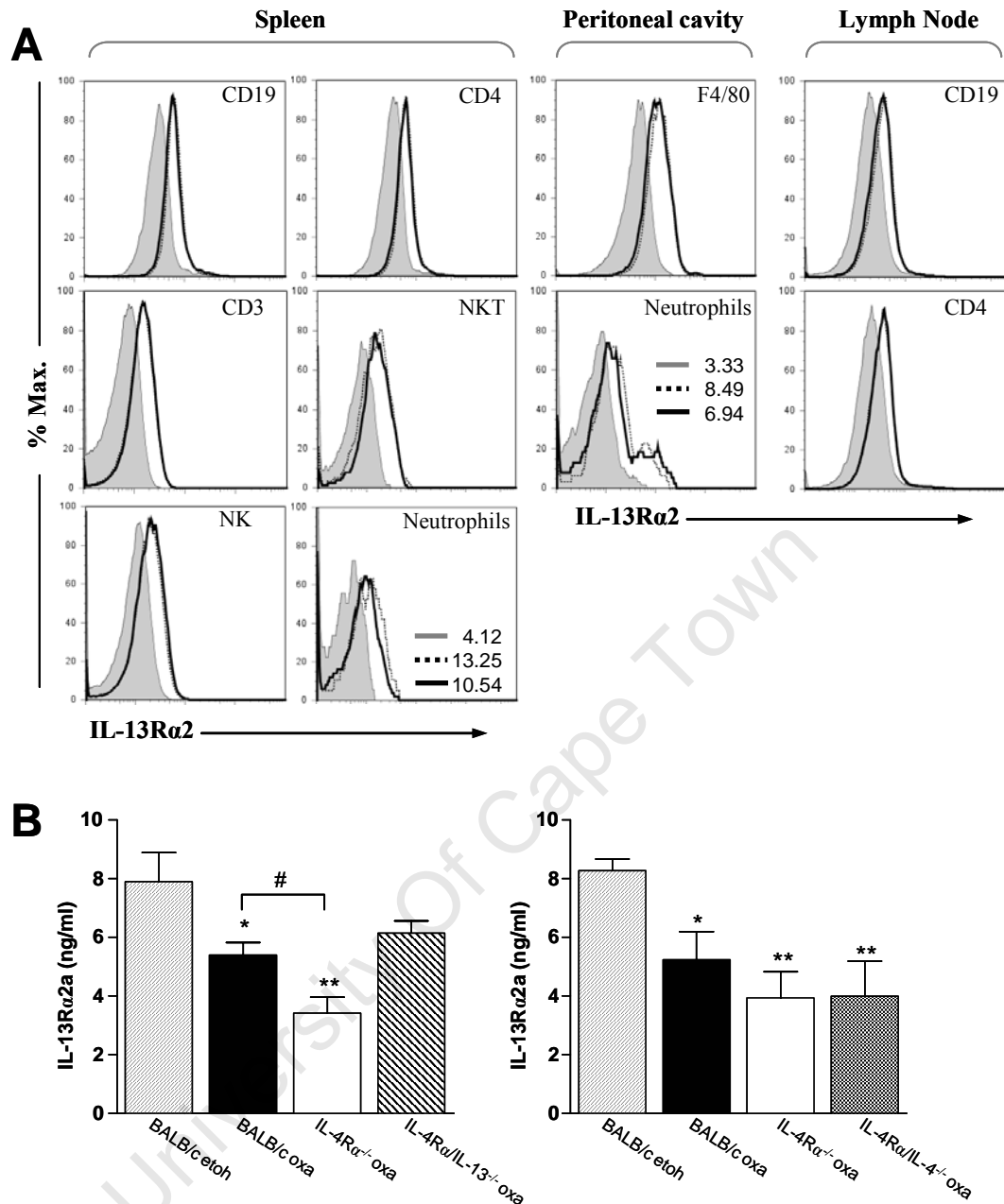
(D) MPO activity in oxazolone-treated IL-4Rα/IL-13<sup>-/-</sup> mice was significantly reduced compared to IL-4Rα<sup>-/-</sup> mice. Data represents 2 individual experiments (n=4-8) with mean values ± SEM and \* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs. BALB/c etoh controls and # = p < 0.05 and ## = p < 0.01, IL-4Rα<sup>-/-</sup> oxa vs. IL-4Rα/IL-13<sup>-/-</sup> oxa mice.

***A role for IL-13 signalling via IL-13R $\alpha$ 2 in oxazolone colitis.***

IL-13R $\alpha$ 2 transcripts have been found in the spleen, liver, lung, thymus and brain (Guo *et al.* 1997; Donaldson *et al.* 1998) and the receptor has been described predominantly as an intracellular molecule in monocytes and respiratory epithelial cells (Daines and Hershey 2002). We used FACS analysis to determine if the IL-13R $\alpha$ 2 was expressed in spleen, peritoneal and lymph node cell populations and if oxazolone-induced colitis had any effect on expression in these cells. Spleen B cells, T cell subpopulations (CD4<sup>+</sup>, CD3<sup>+</sup> and NK T cells) and natural killer cells showed expression of IL-13R $\alpha$ 2 which was unaffected by oxazolone colitis (Figure 9A). Similarly, peritoneal macrophages and T and B cells from the lymph node showed unaffected IL-13R $\alpha$ 2 expression. Interestingly, spleen and peritoneal GR-1<sup>high</sup>/CD11b<sup>+</sup> cells identified as neutrophils (by size and granularity), demonstrated reduced expression of IL-13R $\alpha$ 2 in oxazolone-treated BALB/c mice represented by geometric mean expression (Figure 9A). Pooled spleen and peritoneal cells from individual mice were analysed by FACS and the geometric mean expression of IL-13R $\alpha$ 2 was  $3.42 \pm 0.08$  for BALB/c ethanol-isotype control,  $8.92 \pm 0.58$  BALB/c ethanol-IL-13R $\alpha$ 2 and  $6.89 \pm 0.66$  BALB/c oxazolone-IL-13R $\alpha$ 2 (n=4). A significant reduction (p <0.05) was seen in IL-13R $\alpha$ 2 expression on peritoneal neutrophils of BALB/c mice treated with oxazolone compared with ethanol-only control cells.

The soluble form of IL-13R $\alpha$ 2 has previously been found to be increased in serum of *Schistosoma mansoni* infected mice (Mentink-Kane *et al.* 2004). Therefore, we quantified soluble IL-13R $\alpha$ 2 levels in serum of oxazolone-treated gene deficient mice. Soluble IL-13R $\alpha$ 2 was detected in the serum of control BALB/c mice treated with ethanol-only. This level was significantly reduced in mouse groups that presented with colitis such as, oxazolone-treated BALB/c, IL-4R $\alpha$ <sup>-/-</sup> and IL-4R $\alpha$ /IL-4<sup>-/-</sup> mice. In contrast, IL-4R $\alpha$ /IL-13<sup>-/-</sup> mice protected from colitis and lacking IL-13 production, showed no significant change in soluble IL-13R $\alpha$ 2 levels compared with ethanol-only control mice (Figure 9B).

Together these data demonstrate that expression of the IL-13R $\alpha$ 2 is not limited to specific cell types but rather appears to be widespread and that oxazolone colitis down regulates the expression of IL-13R $\alpha$ 2 on neutrophils.



**Figure 9: IL-13Rα2 expression and secretion in oxazolone-induced colitis**

(A) Spleen cells were CD4<sup>+</sup> or CD3<sup>+</sup> (T cells), CD19<sup>+</sup> (B cells), DX5<sup>+</sup>/CD3<sup>+</sup> (NK T cells), DX5<sup>+</sup>/CD3<sup>-</sup> (natural killer cells) or GR-1<sup>hi</sup>/CD11b<sup>+</sup> (neutrophils). Peritoneal cells were F4/80<sup>+</sup>/CD11b<sup>+</sup> (macrophages) and GR-1<sup>hi</sup>/CD11b<sup>+</sup> (neutrophils). Lymph node cells were CD4<sup>+</sup> or CD19<sup>+</sup> for T and B cells. Groups were, BALB/c ethanol-control-isotype (greyscale), BALB/c ethanol-control-IL-13Rα2 (dotted line) and BALB/c oxa-IL-13Rα2 (black solid line).

(B) Oxazolone colitis was associated with decreased levels of soluble IL-13Rα2 in the serum of susceptible mouse strains. Serum was taken from mice and IL-13Rα2 measured by ELISA. Data represents 2 individual experiments of mice killed on day 3 post-challenge (n=4-8). Mean values are ± SEM and \* = p < 0.05 and \*\* = p < 0.01 vs. BALB/c etoh mice and # = p < 0.05, BALB/c oxa vs. IL-4Rα<sup>-/-</sup> oxa mice.

## DISCUSSION

We show that IL-13 can induce the pathology associated with oxazolone colitis independently of IL-4R $\alpha$ . Oxazolone colitis is a form of experimental colitis mediated by type-2 responses which resemble human ulcerative colitis. Colitis was induced in male BALB/c mice by intrarectal administration of oxazolone subsequent to skin sensitization. Disease was characterized by rapid onset inflammation peaking at day 2 post-challenge and leading to wasting disease and death or complete recovery. Histological assessment demonstrated a superficial colitis with ulceration and an inflammatory infiltrate of lymphocytes and granulocytes. Previous studies show that IL-4 is produced in the initial stages of colitis but is rapidly superseded by IL-13 production and that blocking either one of these cytokines protects C57BL/10 or SJL/J mice from developing disease (Boirivant *et al.* 1998; Heller *et al.* 2002).

The susceptibility of mice to oxazolone-induced colitis is dependent on the strain. Pre-sensitization leads to a prolonged colitis, opposed to severely acute disease resulting in sudden death or quick recovery. The oxazolone model was first described in a highly susceptible SJL/J mouse strain (Boirivant *et al.* 1998) and then established in C57 background strains for the benefit of using genetically manipulated mice (Heller *et al.* 2002; Nieuwenhuis *et al.* 2002). C57/BL6 mice have a tendency towards Th1 immune responses (Charles *et al.* 1999) and therefore require pre-sensitization with oxazolone to induce UC. BALB/c mice are widely recognised as a strain that is predisposed to Th2 immune responses and a number of genetically modified mice are currently available on this background. Oxazolone colitis has been successfully induced in BALB/c mice and the tissue damage (although transient) reflects that of human UC (Kojima *et al.* 2004). We adjusted previous protocols and pre-sensitized BALB/c mice to obtain a reliable model of colitis resembling human ulcerative colitis. By using BALB/c mice lacking or over expressing genes crucial in mediating Th2 responses, such as IL-4, IL-13 and IL-4R $\alpha$  we investigated a role for these genes in the pathology of oxazolone colitis.

The importance of IL-4 and IL-13 signalling has been highlighted in previous oxazolone colitis studies and confirmed by our data using IL-4 and IL-13 deficient and IL-13 transgenic mice. Although blocking IL-4 has previously been shown to protect SJL/J mice from developing oxazolone colitis (Boirivant *et al.* 1998), genetic variability in mice could account for differences in the role of IL-4 in BALB/c mice. Unexpectedly, mice deficient for IL-4R $\alpha$  on all cells developed exacerbated disease in response to

oxazolone. This suggests that IL-4R $\alpha$  plays a regulatory role in colitis and that although IL-4 and IL-13 mediate colitis, the receptor through which they commonly signal is also essential to counterbalance an exaggerated response. The inflammation seen in IL-4R $\alpha^{-/-}$  mice resembled oxazolone treated BALB/c mice with a higher severity. The disease was relatively limited to the distal colon with the microscopic picture showing increased ulceration and epithelial damage. These symptoms were limited to the colon and remained comparable to ulcerative colitis and not a shift towards a type-1 mediated colitis. Furthermore, removing IL-13 but not IL-4 signalling from IL-4R $\alpha$ -deficient mice, significantly protected them compared with wild type BALB/c mice.

Our data suggests that IL-13 has another signalling pathway apart from IL-4R $\alpha$  to mediate oxazolone-induced colitis. Previous studies have shown that the expression of IL-13R $\alpha$ 1 and IL-4R $\alpha$  together was sufficient to render cells responsive to IL-13 (Miloux *et al.* 1997; Hershey 2003). The expression of IL-13R $\alpha$ 2 *in vitro* resulted in high-affinity binding of IL-13 but was insufficient to render cells responsive to IL-13 (Donaldson *et al.* 1998). Furthermore, IL-13R $\alpha$ 2 was found in a soluble form *in vivo* (Zhang *et al.* 1997) and its over expression was shown to diminish IL-13 signalling (Kawakami *et al.* 2001; Daines and Hershey 2002). Together these studies led to speculation that the IL-13R $\alpha$ 2 was a decoy receptor relaying no signal. Recent studies have shown that IL-13 does indeed signal through the IL-13R $\alpha$ 2 by activating AP-1 to induce TGF- $\beta$  secretion (Fichtner-Feigl *et al.* 2006). Therefore the IL-13R $\alpha$ 2 is a good candidate for IL-13 signalling to mediate oxazolone-induced colitis.

The precise mechanism of IL-13 signalling via IL-13R $\alpha$ 2 is unclear. However, macrophages have been shown to produce increased levels of TGF- $\beta$  in response to IL-13R $\alpha$ 2 signalling. Increased levels of TGF- $\beta$  in mice treated with oxazolone protect mice from colitis (Fichtner-Feigl *et al.* 2006). It has also been suggested that because the concentration of TGF- $\beta$  is higher in the proximal colon, oxazolone colitis is limited to the distal colon (Boirivant *et al.* 1998). Considering this data, IL-13 signalling via the IL-13R $\alpha$ 2 should promote protection from disease by increasing TGF- $\beta$ . Furthermore, IL-13R $\alpha$ 2 up regulation has been shown to be dependent on signalling via TNFR1 and IL-4R $\alpha$  (Fichtner-Feigl *et al.* 2006); however we show that IL-13 manages to mediate disease in IL-4R $\alpha^{-/-}$  mice. Previous studies have shown IL-13R $\alpha$ 2 to prolong host survival to *S. mansoni* infection by suppressing chronic type-2-mediated inflammation. Therefore, increased serum levels of IL-13R $\alpha$ 2 were associated with protection from a



type-2-mediated disease (Mentink-Kane *et al.* 2004). Our data shows that reduced serum levels of IL-13R $\alpha$ 2 are associated with disease pathology in oxazolone colitis.

We found IL-13R $\alpha$ 2 expression in many cell types including; T and B lymphocytes, natural killer cells, macrophages and neutrophils. Oxazolone-induced colitis only affected the level of expression of IL-13R $\alpha$ 2 in neutrophils, in which it was reduced. This data suggests a complex role for IL-13 signalling via the IL-13R $\alpha$ 2 in disease. In summary, our results suggest that IL-13/IL-13R $\alpha$ 2 signalling causes disease in oxazolone-induced colitis. Together this data can define a role for IL-13 or IL-13R $\alpha$ 2 and identify specific cell populations involved in the pathology of oxazolone-induced colitis.

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## **2.2 Experimental Study 2**

**IgE production by IL-4 responsive B cells induces  
ulcerative colitis in response to oxazolone**

### ABSTRACT

Oxazolone-induced colitis is a T helper cell type 2 (Th2)-mediated disease which is analogous to human ulcerative colitis (UC). Th2 cytokines interleukin (IL)-4 and IL-13 both signal through a common IL-4 receptor  $\alpha$  chain (IL-4R $\alpha$ ) and are associated with the onset of UC. However, the cell-specific requirements for IL-4R $\alpha$  are unclear. In this study we characterized a novel BALB/c mouse model lacking IL-4R $\alpha$  expression specifically on B cells (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>) to investigate the possible role of IL-4R $\alpha$  responsive B cells during oxazolone colitis. Efficient deletion was confirmed by loss of IL-4R $\alpha$  expression on CD19<sup>+</sup> and B220<sup>+</sup> B cells and impaired IL-4/IL-13 driven antibody response to ovalbumin. In contrast to hemizygous littermate control mice (IL-4R $\alpha$ <sup>-/lox</sup>), which developed severe colitis in response to oxazolone, mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were protected from disease despite increased levels of IL-4 and IL-13. Protection correlated with impaired IgE and IgG1 antibody responses.

Therefore we examined the effect of anti-IgE and anti-FcR $\gamma$ II/III antibodies on BALB/c mice treated with oxazolone. Blocking IgE significantly protected BALB/c mice from the onset of colitis as shown by reduced weight loss, colon shortening, distal colon pathology and death, whereas anti-FcR $\gamma$ II/III treatment had no effect. Furthermore, anti-IgE antibody treatment and protection against oxazolone-induced colitis was associated with significantly reduced mast cell numbers in the distal colon of protected mice. In conclusion, our studies demonstrate that IL-4R $\alpha$  expression on B cells is crucial for driving oxazolone-induced colitis and that pathology is driven by IgE not IgG antibodies.

## INTRODUCTION

Early studies have shown an increase in IgG1 antibody production in ulcerative colitis (UC) patients compared to patients with Crohn's disease (CD) (Kett *et al.* 1987). Conversely, the proportions of IgG2 antibodies were significantly higher in CD patients than in UC. Furthermore, elevated levels of IgE have also been shown in UC patients (Ceyhan *et al.* 2003). Differences in the type of antibody response between the two types of inflammatory bowel disease (IBD) suggest dissimilar mucosal exposure to antigenic stimuli with type 2 antibodies playing a dominant role in UC. Considering the heterogeneity of UC, many aspects of the initial events leading to B cell mediated immunopathology and the mechanisms involving IL-4, IL-13 and type 2 antibody responses remains undefined.

Distinguishing different B cell subpopulations by surface expression of activation markers, identified a unique population of activated CD19<sup>+</sup> B lymphocytes in UC when compared with Crohn's disease (CD) or normal patients (Yacyshyn 1993). This data suggests differing roles for B cells in UC and CD. Furthermore, a striking feature of UC is the substantial and uniform occurrence of IgG producing plasma cells along diseased mucosa (Kett *et al.* 1987). Antibody-mediated hypersensitivity is suggested to be a principal mediator of tissue damage in UC, with IgG<sup>+</sup> plasma cells being of primary pathogenic significance (MacDonald and Monteleone 2006). Although there is a predominant role for IgG antibodies in UC, IgE is known to play an important role in inflammatory reactions. Furthermore, IBD patients particularly with UC, show prevalent allergic symptoms and high IgE levels (Ceyhan *et al.* 2003). The importance of IgE in colitis is further supported by a study using the dextran sulphate sodium (DSS)-induced mouse model of colitis, in which blocking IgE suppressed colitis (Kang *et al.* 2006). These studies suggest that IgE-dependent mechanisms may be involved in the pathogenesis of UC.

IgE binds to the FcεRI on the surface of mast cells (Akdis 2006), the second exposure to antigen leads to cross-linking of bound IgE resulting in mast cell degranulation releasing potent inflammatory mediators (Sampson 1999; van Drunen and Fokkens 2006). Intestinal mast cell numbers have been reported to be either increased or decreased in patients with ulcerative colitis. These discrepancies have been suggested to be due to differences in methods of preparing tissue sections or due to degranulation of activated mast cells (Bischoff *et al.* 1996). However, elevated levels of mast cell-derived

mediators, such as tryptase and TNF- $\alpha$ , suggest mast cell activation by IgE cross-linking in UC (Bischoff *et al.* 1996; Raithel *et al.* 2001). IL-4 and possibly IL-13 induce IgE isotype switching in mouse B cells via the IL-4R $\alpha$  (Finkelman *et al.* 1988; Bonnefoy *et al.* 1996; Lai and Mosmann 1999; Brombacher 2000). Deleting IL-4R $\alpha$  specifically from B cells would determine a role for IL-4/IL-13 responsive B cells in oxazolone-induced colitis.

IL-4 and IL-13 are closely related cytokines with pleiotropic immune functions mediated through a shared receptor subunit, IL-4R $\alpha$  (Brombacher 2000). The IL-4R $\alpha$  chain, in association with the gamma common ( $\gamma c$ ) chain, binds IL-4 with high affinity. It also functions as a component of the IL-13 receptor by binding with IL-13R $\alpha 1$  (Nelms *et al.* 1999). Both cytokines are involved in mechanisms promoting UC (Boirivant *et al.* 1998; Kojima *et al.* 2004; Wang *et al.* 2004) and allergic pathology (Grunig *et al.* 1998; Wills-Karp *et al.* 1998). Therefore, IL-4R $\alpha$  mediates tissue damage or confers immunity depending on cell type-specific expression or the type of stimulus (Brombacher 2000).

The cellular contributions of IL-4R $\alpha$  in inflammatory disease can be dissected by using mice deficient in IL-4R $\alpha$  expression on specific cell types. In this study, we addressed the role of B cell IL-4R $\alpha$  expression in UC. This was achieved by generating a B cell-specific IL-4R $\alpha$ -deficient mouse (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>). Our results demonstrate the successful generation of transgene-bearing mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> BALB/c mice that have effective impairment of IL-4R $\alpha$  specifically on B cells. We used a mouse model of allergic asthma to functionally characterize mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice and showed that mice sensitized with ovalbumin lack type 2 antibody responses but remain susceptible to ovalbumin-induced allergic asthma. This data suggests signalling via the IL-4R $\alpha$  on B cells is required for type 2 antibody production. Similarly, mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice treated with oxazolone to induce a Th2-mediated ulcerative colitis also had disrupted type 2 antibody responses. However, these mice were significantly protected from colitis suggesting the involvement of IgE or IgG1 in ulcerative colitis. Subsequently we showed that, BALB/c mice treated with anti-IgE but not anti-Fc $\gamma$ RII/III antibodies were protected from oxazolone-induced colitis with a reduction in mast cell numbers. Therefore, IgE production by IL-4 responsive B cells induces ulcerative colitis in response to oxazolone.

## MATERIALS AND METHODS

### ***Generation and genotyping of mb1<sup>cre</sup> IL-4Rα<sup>-lox</sup> BALB/c mice.***

Transgenic mb1<sup>cre</sup> mice (Hobeika *et al.* 2006) were first backcrossed to BALB/c for nine generations and then intercrossed with IL-4Rα-deficient (IL-4Rα<sup>-/-</sup>) and IL-4Rα<sup>lox/lox</sup> mice to generate mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> BALB/c mice. Transgene negative littermates (IL-4Rα<sup>-lox</sup>) were used as controls in all experiments. Specific PCR primer pairs were used to confirm the genotype of mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice. IL-4Rα: 5'-TGACC-TACAAGGAACCCAGGC-3' and 5'-CTCGGCGCACTGACCCATCT-3'; deletion: 5'-GGCTGCTGACCTGGAATAACC-3' and 5'-CCTTTGAGAACTGCGGGCT-3'; LoxP: 5'-CCCTTCCTGGCCCTGAATTT-3' and 5'-GTTTCCTCCTACCGCTGATT-3' hCre: 5'-ACCTCTGATGAAGTCAGGAAGAAC-3' and 5'-GGAGATGTCCTTCACTCTGATTCT-3'. PCR conditions were as follows: 94°C/1min, 94°C/30sec, 55°-60°C/20sec and 72°C/1min for 40 cycles on a MJ thermocycler (Biozym, Hessisch Oldendorf, Germany). Routine genotyping was performed by Wendy Green and Rayaana Fredericks in the Division of Immunology. Male mice on a BALB/c background were used for oxazolone experiments, while sex matched mice were used for characterization and allergy experiments. Mice (aged 6-10 weeks) were bred and housed in specific pathogen-free conditions at the University of Cape Town, South Africa. All experiments were approved by the University's Animal Ethics Committee.

### ***Efficiency of IL-4Rα deletion by Real-Time PCR.***

Genomic DNA was prepared from CD3<sup>+</sup>CD19<sup>-</sup> and CD3<sup>-</sup>CD19<sup>+</sup> sorted lymph node cells (>99% purity) from mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup>, IL-4Rα<sup>-lox</sup> and IL-4Rα<sup>-/-</sup> mice using a FACsvantage flow cytometer (BD biosciences, Ferndale, South Africa). A standard curve was prepared of serial 10-fold dilutions from DNA product using primers specific for IL-4Rα exon 5 (control) and exon 8 (deleted by hCre expression). Primers: exon 5 forward: 5'-AACCTGGGAAGTTGTG-3' and reverse: 5'-CACAGTTCCATCTGGTAT - 3'; exon 8 forward: 5'-GTACAGCGCACATTGTTTTT-3' and reverse 5'-CTCGGCGCACTGACCCATCT-3'. (See Appendix A for solutions and supplementary methods)

### ***Flow Cytometry.***

IL-4Rα surface expression was detected on spleen or lymph node cells by phycoerythrin (PE) or biotinylated anti-CD124 (IL-4Rα, clone: M-1). Cell subpopulations were identified with fluorescein isothiocyanate (FITC), PE or biotinylated mAbs against:



CD19 (clone: 1D3), B220 (clone: RA3-6B2), CD3 (clone: 145-2C11), CD4 (clone: GK1.5), CD8 (clone: 53.6.72), pan-natural killer (clone: DX5), CD11b (clone: M1/70) and F4/80 (clone: A3-1, Serotec, Oxford UK). Surface expression of the low affinity IgE receptor, CD23 (clone: B3B4) was analysed on CD3<sup>+</sup> and CD19<sup>+</sup> cells from spleen, lymph node and lung suspensions. Biotin-labelled antibodies were detected by streptavidin-allophycocyanin (APC) and dead cells were excluded from analysis by 7-AAD staining (Sigma). Antibodies were from BD Pharmingen (San Diego, CA) except where noted otherwise. The anti-FcR (clone: 2.4G2) was used in order to block non-antigen-specific binding of immunoglobulins to the FcγII and FcγIII receptors. Cells were acquired using a FACSCalibur (Beckton-Dickinson, Ferndale, South Africa) and data were analysed with Cellquest (BD biosciences) or Flowjo software (Treestar).

### ***T cell proliferation.***

Lymph node CD4<sup>+</sup> T cells were positively selected using anti-CD4 coupled to microbeads (MACS, Miltenyi Biotec) to a purity of >90% as confirmed by FACS. Single cell suspensions prepared from lymph nodes were incubated with anti-CD4 microbeads and passed through an LS separation column (max 10<sup>8</sup> cells). The magnetically labelled CD4<sup>+</sup> cells were retained in the column while unlabeled cells ran through. After removal from the magnetic field, CD4<sup>+</sup> cells were eluted and viable cells counted using Trypan Blue exclusion (Sigma). Triplicates of 5x10<sup>5</sup> cells/ml were stimulated with serial dilutions of IL-4 or IL-2 (BD Pharmingen) in complete Iscoves Modified Dulbeccos Medium (IMDM) supplemented with 10% FCS (Gibco Life Technologies, Paisly, UK), penicillin, streptomycin, 1mM sodium pyruvate, 50 μM B2-ME (Sigma) and NEAA (Invitrogen). After 48h of incubation at 37°C and 5% CO<sub>2</sub>, cells were pulsed with 1μCi [<sup>3</sup>H] thymidine (AEC Amersham, Uppsala, Sweden) and incubated for a further 18h. [<sup>3</sup>H] incorporation was measured in a liquid scintillation counter and results were expressed as counts per minute (cpm).

### ***In vitro Th2 differentiation.***

Positively selected CD4<sup>+</sup> T cells, enriched to a purity of >90% (described above) were used for *in vitro* Th1/Th2 differentiation as previously described (Mohrs *et al.* 2000; Herbert *et al.* 2004; Radwanska *et al.* 2007; Dewals *et al.* 2009). Briefly, 1x10<sup>6</sup> cells/ml were cultured on anti-CD3 (clone: 145-2C11, 10μg/ml) and anti-CD28 (clone: 37.51, 5μg/ml) coated plates. T helper development was driven by IL-12 (5ng/ml) or IL-4

(50ng/ml) and neutralising antibody against IL-4 (clone: 11B11, 10µg/ml) or IFN-γ (clone: An18KL6, 50µg/ml). After 72h, the cells were washed and supplemented with new media containing the above stimulants including IL-2 (20U/ml). After 48h the cells were washed extensively, transferred to fresh anti-CD3 coated microwell plates and cultured in IL-2 (20U/ml). Supernatants were collected after 48h of culture and cytokine production measured by enzyme-linked immunosorbent assay (ELISA). All stimulants were from BD Pharmingen unless stated otherwise.

### ***Sensitization and challenge protocols.***

To confirm the functional disruption of IL-4Rα specifically on B cells, mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup>, IL-4Rα<sup>-lox</sup> and IL-4Rα<sup>-/-</sup> mice were sensitized by intraperitoneal inoculation of 50µg ovalbumin in 1.5% aluminium hydroxide adjuvant (alum) (both Sigma-Aldrich, Germany). Mice were boosted at days 7 and 14, challenged by intranasal administration of 100µg ovalbumin at days 21, 22 and 23 and killed at day 25 for analysis. Control mice were treated with PBS-alum only. Oxazolone-mediated colitis was induced in BALB/c mice by modifying a previously described method (Neurath *et al.* 2002). Anesthetized mice were sensitized on the shaved abdomen by application of 3% oxazolone (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one; Sigma-Aldrich) in 100% ethanol (150µl) followed 7 days later by intrarectal administration of 1% oxazolone in 50% ethanol (150µl). Control mice were sensitized and challenged with ethanol only. Mice were killed 7 days post-challenge and immunopathological characterization was performed. To determine the role of IgE or IgG isotypes in oxazolone-induced colitis, *in vivo* neutralization was performed with 2 doses of anti-IgE (84.1C), anti-FcγRII/III (2.4G2) or rat anti-IgG control (afrc-mac51) (200µg/mouse), before sensitization and challenge with oxazolone (Figure 6A).

### ***Disease activity index (DAI).***

Oxazolone treated BALB/c mice develop rapid-onset colitis marked by weight loss and distress. To monitor disease progression, mice were weighed and monitored for distress and gastrointestinal symptoms 1-2 times daily. Weight loss was measured as a percentage of starting weight and distress was scored at day 2 post-challenge according to appearance, clinical signs and natural or provoked behaviour (Wolfensohn and Lloyd 1998). Mice were scored on a scale of 0-16 and killed when the most severe score (16) was reached or ≥20% body weight was lost. Mortality rates were monitored in a study of

10 mice/ group and the same criteria were monitored as mentioned above. Colon length was measured from the anus to the caecum and recorded as an indication of inflammation.

### ***Histological analysis***

Lung tissue or distal colon sections taken 1cm from the anus were fixed in ice cold 4% phosphate-buffered formalin to prevent tissue autolysis. Paraffin embedded tissue was cut into 2µm sections and stained with haematoxylin and eosin (H&E) for inflammatory cells, Periodic acid-Schiff reagent (PAS) for mucus producing goblet cells or toluidine blue for mast cells. Histopathological grading of oxazolone-induced colitis was adjusted from a previous study (Iijima *et al.* 2004) as described in chapter 2. Essentially, mice were graded on 5 criteria, 1) presence of mononuclear cells, 2) reduced goblet cells, 3) epithelial injury, 4) granulocyte infiltration and 5) oedema. Each criteria were scored from 0-3 and the total score was added resulting in a total additive score of between 0 (no colitis) and 15 (maximal colitis activity). Histology sections were processed and stained by the Department of Surgery, Groote Schuur Hospital.

### ***Antibody detection by ELISA.***

Blood samples were taken by tail vein bleeding or directly from mice after killing and collected in gel separation tubes (Microtainer, BD, USA). Samples were centrifuged at 4500rpm for 20min at 4°C. Ovalbumin-specific IgG1, IgG2a and IgG2b were measured by indirect ELISA using 5µg/ml ovalbumin for coating (overnight at 4°C) on nunc maxisorb ELISA plates (Nunc, Reskilde Denmark). The plates were blocked for 3h at 37°C, washed 3x, serum added in serial dilutions and incubated overnight at 4°C. Alkaline Phosphatase (AP)-labelled goat-anti-mouse isotype-specific Abs (Southern Biotechnology, Birmingham, USA) were added for 1h at 37°C. Total IgE was measured by sandwich ELISA using clone 84.1C as a coat and anti-mouse IgE (Southern Biotechnology, USA) for detection (Nieuwenhuizen *et al.* 2007). Total IgE, IgG1, IgG2a and IgG2b levels were measured for naïve and oxazolone-treated mice (days 3 and 7) by sandwich ELISA. Anti-mouse IgE (84.1C), IgG1, IgG2a and IgG2b mAbs were used for coating, purified recombinant mouse IgE, IgG1, IgG2a and IgG2b (BD Pharmingen) were used as standards and anti-mouse isotype-specific Abs (Southern Biotechnology, USA) for detection. 4-Nitrophenylphosphate (PNP, Fluka, Switzerland) dissolved at

1mg/ml in substrate buffer was added to all samples and the absorbance measured at 405nm on a Versamax microplate spectrophotometer (Molecular Devices, Germany).

***Cellular restimulation and cytokine detection by ELISA.***

Mediastinal lymph node cells from ovalbumin sensitized mice were incubated at  $1 \times 10^6$  cells/ml on anti-CD3 coated plates (clone: 145-2C11, 10 $\mu$ g/ml) in IMDM with 10%FCS for 48h at 37°C and 5% CO<sub>2</sub>. Supernatants were collected and used for sandwich ELISA detection of cytokine levels (as above). Plates were coated with purified anti-IL-4 (clone: 11B11, 2 $\mu$ g/ml), anti-IFN- $\gamma$  (clone: An18KL6, 1 $\mu$ g/ml) or anti-IL-13 (clone: 38213, 1 $\mu$ g/ml, BD Pharmingen) antibodies, blocked and serially diluted standards (purified recombinant IL-4, IFN- $\gamma$  or IL-13) or cell supernatants were added. Biotinylated anti-mouse secondary antibodies for IL-4 (clone: BVD6-24G2), IFN- $\gamma$  (clone: XMG1.2) or IL-13 (goat anti-mouse, 0.5 $\mu$ g/ml, BD Pharmingen) were added for 3h at 37°C followed by horseradish peroxidase (HRP) labelled streptavidin. Subsequently, plates were developed with TMB Peroxidase Substrate (KPL, USA), the colour reaction stopped with 2M H<sub>2</sub>SO<sub>4</sub> and absorption measured at 450nm.

***Cytokine detection in colon tissue homogenates by ELISA.***

Colon sections were pooled from oxazolone-treated and control mice, snap frozen in liquid nitrogen and stored at -80°C until analysis. Tissue sections were homogenized in PBS supplemented with protease inhibitors (Sigma, Germany) and normalised to 10mg/ml protein using BCA protein assay kit (Pierce, Rockford, IL). IL-4, IL-13 and IFN- $\gamma$  cytokines were measured as described above.

***Statistical analysis.***

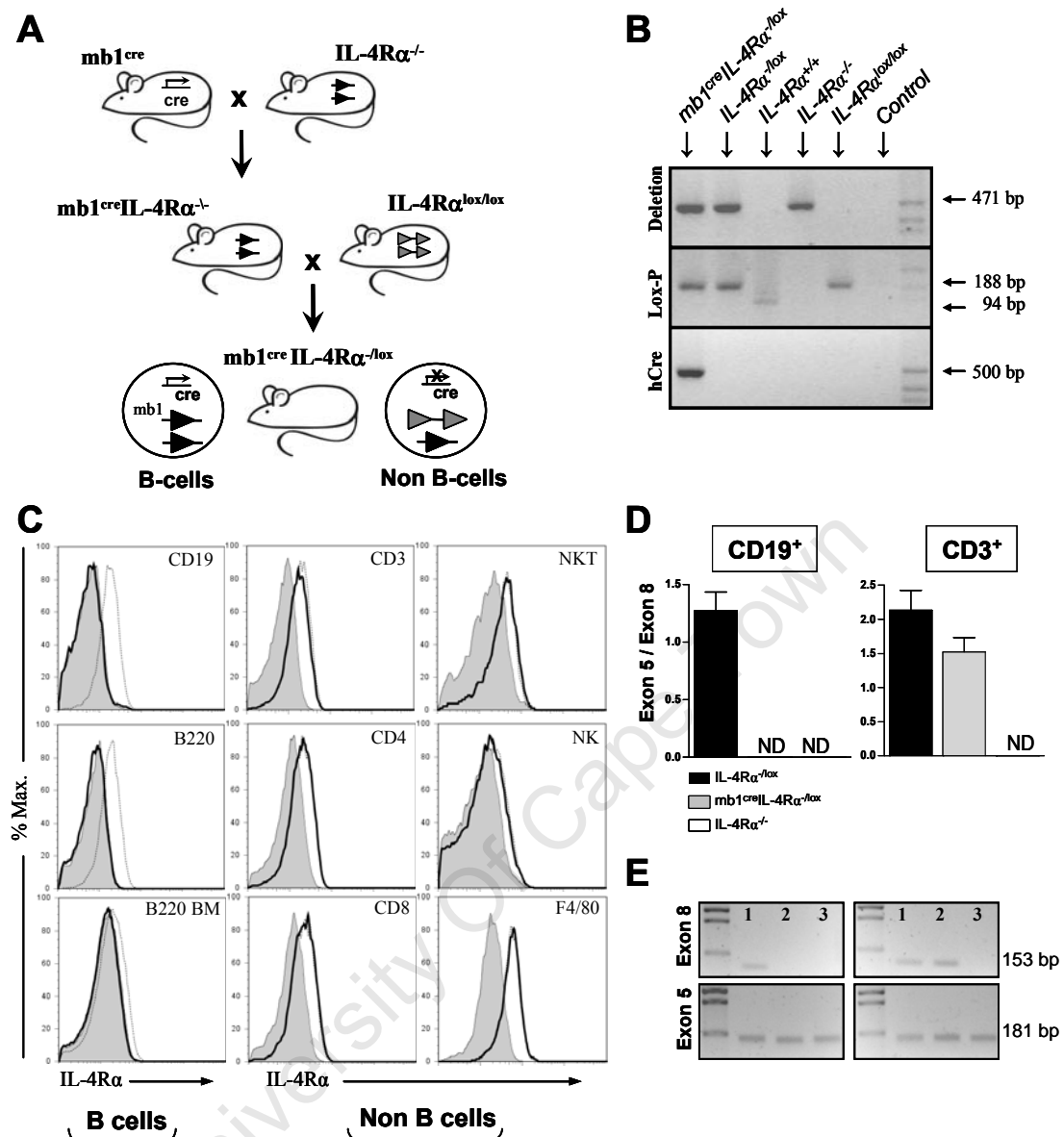
Values are given as mean  $\pm$  SEM, and significant differences were determined using unpaired two-tailed students *t* test or one-way ANOVA using a Bonferroni post-test (GraphPad Prism). Values of  $p < 0.05$  were considered significant.

## RESULTS

### ***B cell-specific IL-4R $\alpha$ deletion in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> BALB/c mice.***

Conditional B cell-specific IL-4R $\alpha$ -deficient BALB/c mice were generated by the Division of Immunology genotyping group as previously described for, macrophages/neutrophils and T cell subpopulations (Herbert *et al.* 2004; Radwanska *et al.* 2007; Dewals *et al.* 2009). Essentially, global IL-4R $\alpha$ <sup>-/-</sup> mice (Mohrs *et al.* 1999) were intercrossed with transgenic mb1<sup>cre</sup> (Hobeika *et al.* 2006) and IL-4R $\alpha$ <sup>lox/lox</sup> mice (Figure 1A). Transgene-bearing B cell-specific IL-4R $\alpha$ -deficient (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) mice were identified by PCR genotyping (Figure 1B). Primers targeting intron 6 and intron 10 of IL-4R $\alpha$ , amplified product (471bp) in IL-4R $\alpha$ -deleted mice (Deletion). In BALB/c mice, the targeted region was too large for PCR amplification. Mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>, IL-4R $\alpha$ <sup>-lox</sup> and global IL-4R $\alpha$ <sup>-/-</sup> mice were positive for IL-4R $\alpha$  deletion. Primers designed to flank the loxP insertion site amplified a 188bp product in “floxed” mice or a 94bp product in wild type BALB/c (IL-4R $\alpha$ <sup>+/+</sup>) mice. The inserted hCre-transgene was amplified (500bp) in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> but not IL-4R $\alpha$ <sup>-lox</sup>, BALB/c or global IL-4R $\alpha$ <sup>-/-</sup> mice. These data confirm the successful generation of hCre transgenic, loxP flanked, B cell-specific IL-4R $\alpha$  disrupted mice.

FACS analysis of IL-4R $\alpha$  surface expression confirmed efficient deletion on B cells (CD19<sup>+</sup> or B220<sup>+</sup>) isolated from spleen and bone marrow from mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice compared with IL-4R $\alpha$ <sup>-lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> controls. The cellular specificity of IL-4R $\alpha$  deletion was confirmed because splenic T cells (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> or NKT cells), natural killer cells (DX5<sup>+</sup>) and peritoneal macrophages (F4/80<sup>+</sup>) maintained IL-4R $\alpha$  expression (Figure 1C). To establish the efficiency of IL-4R $\alpha$  deletion at the genomic level from high purity ( $\geq 99\%$ ) FACS sorted cells; Real-Time quantitative PCR reactions were designed. Primers amplifying genomic regions of the IL-4R $\alpha$  gene, affected or unaffected by the deletion were used (Radwanska *et al.* 2007). The number of exon 8 alleles (one copy in <sup>-lox</sup> and absent in <sup>-/-</sup> mice) relative to exon 5 alleles (both present in all cells) of IL-4R $\alpha$  were determined in CD19<sup>+</sup> and CD3<sup>+</sup> cells (Figure 1D). Confirming FACS analysis, efficient deletion of loxP-flanked IL-4R $\alpha$  exon 8 was observed in CD19<sup>+</sup> B cells from mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice. As expected, no exon 8 product was visible in CD19<sup>+</sup> and CD3<sup>+</sup> cells of global IL-4R $\alpha$ <sup>-/-</sup> mice, whereas an equivalent ratio of exon 8 vs. exon 5 was maintained from cells of IL-4R $\alpha$ <sup>-lox</sup> mice (Figure 1D).



**Figure 1: Generation of mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice.**

(A) Mouse breeding strategy. IL-4Rα<sup>-/-</sup> BALB/c mice were intercrossed with transgenic BALB/c mice expressing Cre-recombinase under control of the *mb-1* promoter and IL-4Rα<sup>lox/lox</sup> mice to generate mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice. The “floxed” IL-4Rα allele (grey arrows) and deleted allele (black arrows) are shown.

(B) Genotyping of transgene bearing B cell-specific IL-4Rα-deficient mice. The deleted IL-4Rα PCR yields a product of 471bp, loxP, 188bp (floxed), or 94bp (wild-type) and *hCre*-specific a 500bp product.

(C) Phenotypic analysis. Spleen, bone marrow (BM) or peritoneal cells from naïve IL-4Rα<sup>-lox</sup> (dotted line), IL-4Rα<sup>-/-</sup> (greyscale) and mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> (bold line) mice were stained for the surface expression of IL-4Rα. B cells were CD19<sup>+</sup>/CD3<sup>-</sup> or B220<sup>+</sup>/CD3<sup>-</sup>, T cells were CD4<sup>+</sup>/CD3<sup>+</sup> or CD8<sup>+</sup>/CD3<sup>+</sup>, natural killer T cells were DX5<sup>+</sup>/CD3<sup>+</sup>, natural killer cells were DX5<sup>+</sup>/CD3<sup>-</sup>, and macrophages were CD11b<sup>+</sup>/F4/80<sup>+</sup>. Data are representative of 3 independent experiments.

(D) Efficiency of IL-4Rα-deletion. The ratio of IL-4Rα exon 5 and exon 8 alleles was determined by real-time PCR from genomic DNA purified from CD19<sup>+</sup> or CD3<sup>+</sup> cells. ND = samples not detected.

(E) PCR products were visualized on an agarose gel. 1 = IL-4Rα<sup>-lox</sup>, 2 = mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> and 3 = IL-4Rα<sup>-/-</sup>. Data represents 2 independent experiments with triplicate values ± SEM.

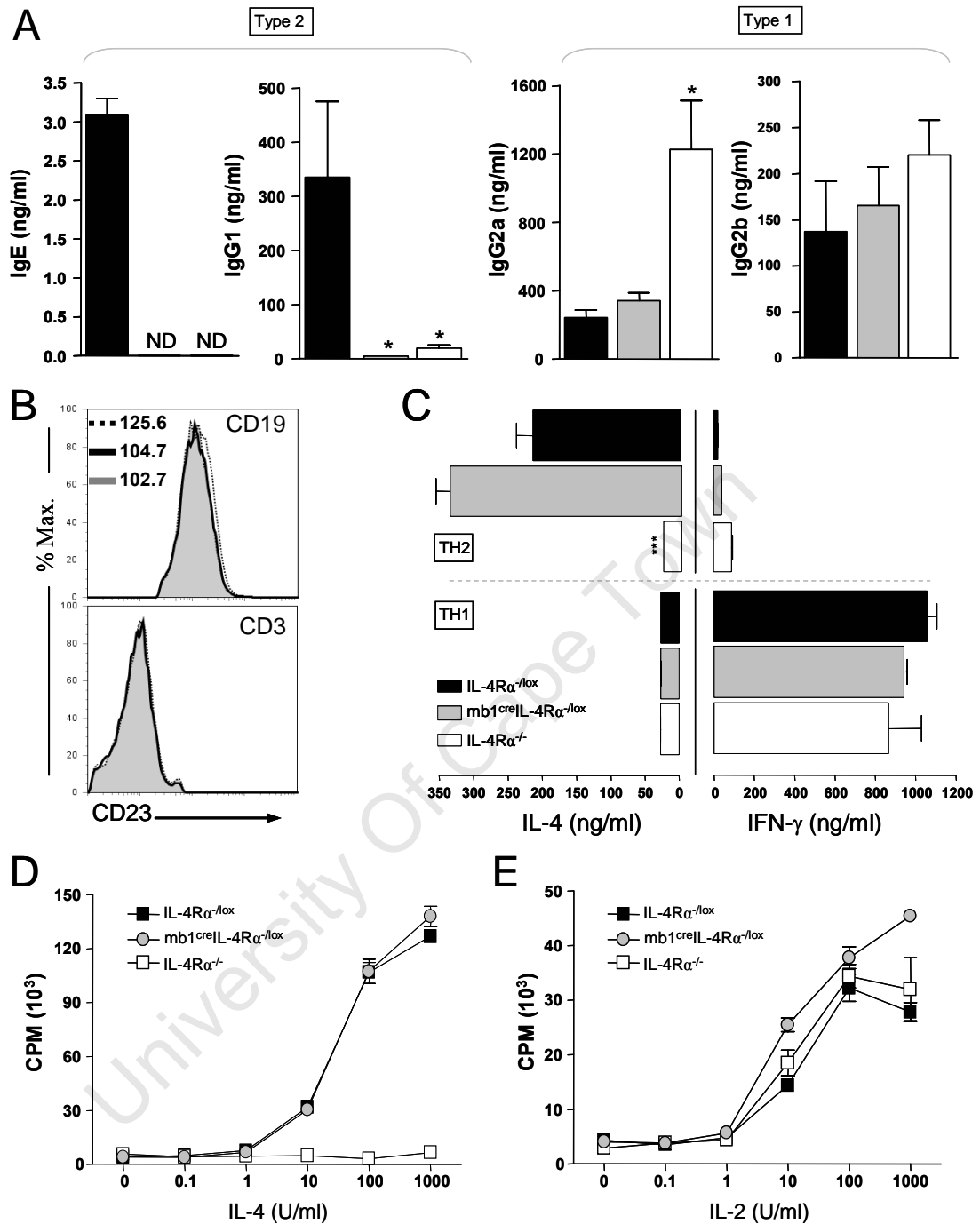
PCR product size was confirmed on an agarose gel (Figure 1E). These data provide evidence of efficient deletion of IL-4R $\alpha$  restricted to CD19<sup>+</sup> B cells from mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice.

### ***Selective impairment of IL-4R $\alpha$ function in B cells.***

Th2 cells stimulate IL-4 responsive B cells to produce IgE or IgG1 antibodies. To determine if the absence of IL-4 signalling affects antibody production in naïve mice, serum was analysed for IgE, IgG1, IgG2a and IgG2b antibodies. Figure 2A shows that IL-4 responsive B cells are required for IgE and IgG1 production in both mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice and that without this signalling global IL-4R $\alpha$ <sup>-/-</sup> mice have an increase in naïve levels of IgG2a. CD23 is the low affinity receptor for IgE and requires IL-4 to be expressed on mature B cells (Kijimoto-Ochiai 2002). FACS analysis showed the surface expression of this receptor to be reduced in the absence of IL-4R $\alpha$  signalling on B cells, even in naïve mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice compared with IL-4R $\alpha$ <sup>-/lox</sup> mice (Figure 2B). These data suggest the functional disruption of IL-4R $\alpha$  on B cells of mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice comparable to global IL-4R $\alpha$ -deficient mice.

IL-4 also induces the differentiation of naïve T cells into Th2 cells *in vitro* (Le Gros *et al.* 1990), while IL-12 drives Th1 development (Seder *et al.* 1993). We wanted to test whether deletion of IL-4R $\alpha$  on B cells or the insertion of loxP sites on all cells had an effect on CD4<sup>+</sup> T cell function. Therefore, naïve CD4<sup>+</sup> T cells were stimulated with anti-CD3/CD28 and treated with IL-12/anti-IL-4 to induce Th1 development or IL-4/anti-IFN- $\gamma$  to induce Th2 development. T cells were able to differentiate into Th1 or Th2 cells in both IL-4R $\alpha$ <sup>-/lox</sup> and mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice (Figure 2C). This was in contrast to IL-4R $\alpha$ <sup>-/-</sup> mice, which only responded to IL-12 treatment.

IL-4 also induces proliferation of naïve CD4<sup>+</sup> T cells (Seder and Paul 1994). Naïve CD4<sup>+</sup> T cells from IL-4R $\alpha$ <sup>-/lox</sup>, IL-4R $\alpha$ <sup>-/-</sup> and mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> BALB/c mice were stimulated with increasing doses of IL-4 or IL-2. DNA synthesis was measured by [<sup>3</sup>H] thymidine incorporation and showed that CD4<sup>+</sup> T cells from IL-4R $\alpha$ <sup>-/lox</sup> and mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were able to proliferate in response to IL-4 in contrast to global IL-4R $\alpha$ <sup>-/-</sup> mice (Figure 2D). Impairment of IL-4 signalling was IL-4R $\alpha$  specific, as proliferative responses to IL-2, which shares a  $\gamma$ c-chain with type I IL-4R, were unaffected in all three strains (Figure 2E). These results confirm an unimpaired response of CD4<sup>+</sup> T cells to IL-4 in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice.



**Figure 2: Functional characterization of naïve mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice.**

(A) Impaired Type 2 antibody production. Total antibody levels were determined in the serum of naïve mice by ELISA and mean  $\pm$  SEM.

(B) The low affinity IgE receptor (CD23) expression was determined by FACS analysis on naïve lymph node T and B cells. Geometric means are given of IL-4Rα<sup>-lox</sup> (dotted line), IL-4Rα<sup>-/-</sup> (greyscale) and mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> (bold line) mice.

(C) Normal Th2 differentiation. Th1 versus Th2 differentiation was promoted from naïve CD4<sup>+</sup> T cells with IL-4/anti-IFN-γ or IL-12/anti-IL-4 mAbs. IFN-γ or IL-4 production was measured by ELISA. Values represent mean  $\pm$  SEM from triplicate cultures.

(D) CD4<sup>+</sup> lymph node cells from naïve IL-4Rα<sup>-lox</sup>, IL-4Rα<sup>-/-</sup> and mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice were stimulated with serial dilutions of IL-4 or (E) IL-2 (control). DNA synthesis was measured by [<sup>3</sup>H] thymidine incorporation. Data represents 2 independent experiments with \* = p < 0.05 and \*\*\* = p < 0.001 vs. IL-4Rα<sup>-lox</sup> mice and samples not detected (ND) are indicated.

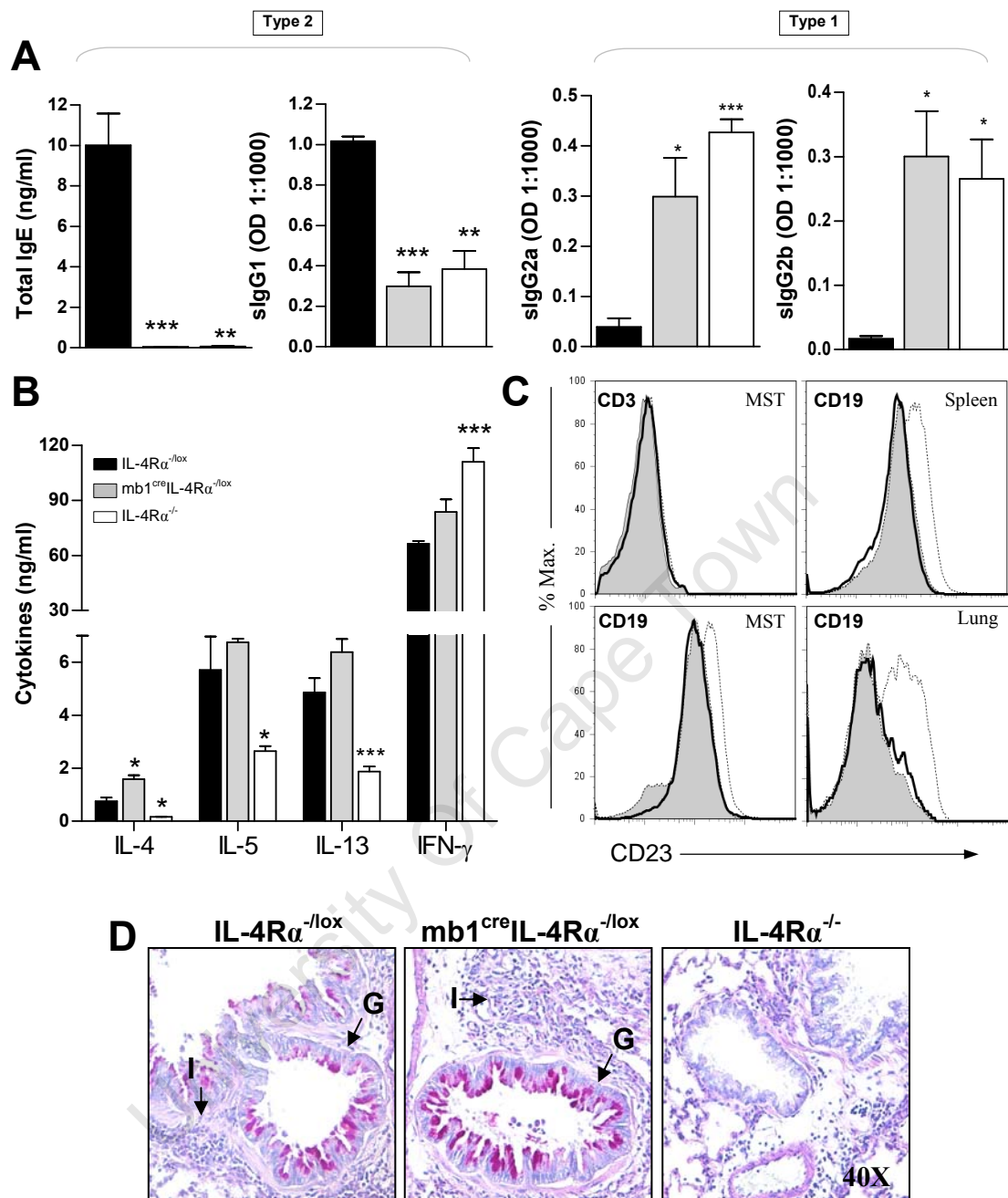


***B cell-specific IL-4R $\alpha$ -deficient mice develop ovalbumin-induced allergy.***

IgE and IgG1 antibody secretion is dependant on IL-4 signalling on B lymphocytes (Shimoda *et al.* 1996). To show abrogated function of IL-4R $\alpha$  on B lymphocytes, mice were sensitized with ovalbumin-alum at weekly intervals for 3 weeks and challenged intranasally with 100 $\mu$ g ovalbumin. Total serum IgE titres and antigen-specific IgG1, IgG2a and IgG2b were measured by ELISA. Mice treated with alum-only were included as a control and antibody levels were subtracted from experimental mouse groups. B cell-specific IL-4R $\alpha$ -deficient mice had significantly reduced type 2 antibody levels (IgE and IgG1) with a shift towards type 1 antibody response (IgG2a and IgG2b), comparable to global IL-4R $\alpha$ <sup>-/-</sup> mice (Grunewald *et al.* 1998) (Figure 3A). As expected IL-4R $\alpha$ <sup>-lox</sup> mice maintained a type 2 polarized antibody response to ovalbumin.

Development of allergic asthma is driven by Th2 cytokines, especially IL-4, IL-5 and IL-13. Therefore, mediastinal lymph nodes from ovalbumin-treated mice were restimulated with anti-CD3 and analyzed for cytokine production. Despite the reduced type 2 antibody response by mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice, they maintained a Th2 polarized cytokine response with increased levels of IL-4, IL-5 and IL-13 comparable to IL-4R $\alpha$ <sup>-lox</sup> (Figure 3B). Supporting the increased levels of type 1 antibodies, IL-4R $\alpha$ <sup>-/-</sup> mice had significantly higher IFN- $\gamma$  but reduced IL-4, IL-5 and IL-13 (Figure 3B). As the low affinity IgE receptor (CD23) is expressed on IL-4-activated B cells (Kijimoto-Ochiai 2002), we wanted to determine if the lack of IL-4 signalling on mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> mice would effect CD23 surface expression by FACS analysis. Mesenteric lymph node, spleen and lung CD19<sup>+</sup> cells showed a reduced surface expression of CD23 when compared with IL-4R $\alpha$ <sup>-lox</sup> mice (Figure 3C). No differences were detected in the surface expression on CD3<sup>+</sup> lymph node cells in all three groups (Figure 3C).

Lung histopathological analysis showed ovalbumin induced mucus hypersecretion by goblet cells and cellular infiltration in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> and IL-4R $\alpha$ <sup>-lox</sup> mice, both common signs of allergic airway inflammation (Figure 3D). In IL-4R $\alpha$ <sup>-/-</sup> mice, allergic reactions were completely abolished with no mucus production and low levels of cellular infiltration in the lungs, comparable to PBS/alum only control mice (Grunig *et al.* 1998) (Figure 3D). These results show that although B cell-specific IL-4R $\alpha$ -deficient mice have an abrogated type 2 antibody response, they remain susceptible to ovalbumin-induced allergic asthma.



**Figure 3: Impaired type 2 antibody response in an ovalbumin allergy model.**

(A) Mice were sensitized by i.p. injection for 3 weeks with ovalbumin and challenged by intranasal inoculation. Total IgE (ng/ml) or antigen-specific IgG1, IgG2a and IgG2b (OD at 1:1000) were measured by ELISA after killing the mice on day 25.

(B) Mediastinal lymph nodes were restimulated with anti-CD3 and cytokine concentrations measured in the supernatant.

(C) Impaired surface expression of the low affinity IgE receptor (CD23) was measured by FACS analysis on CD3<sup>+</sup> and CD19<sup>+</sup> mediastinal lymph node (MST), spleen and lung cells. IL-4R $\alpha^{-/-}$  (dotted line), IL-4R $\alpha^{-/-}$  (greyscale) and mb1<sup>cre</sup>IL-4R $\alpha^{-/-}$  (bold line).

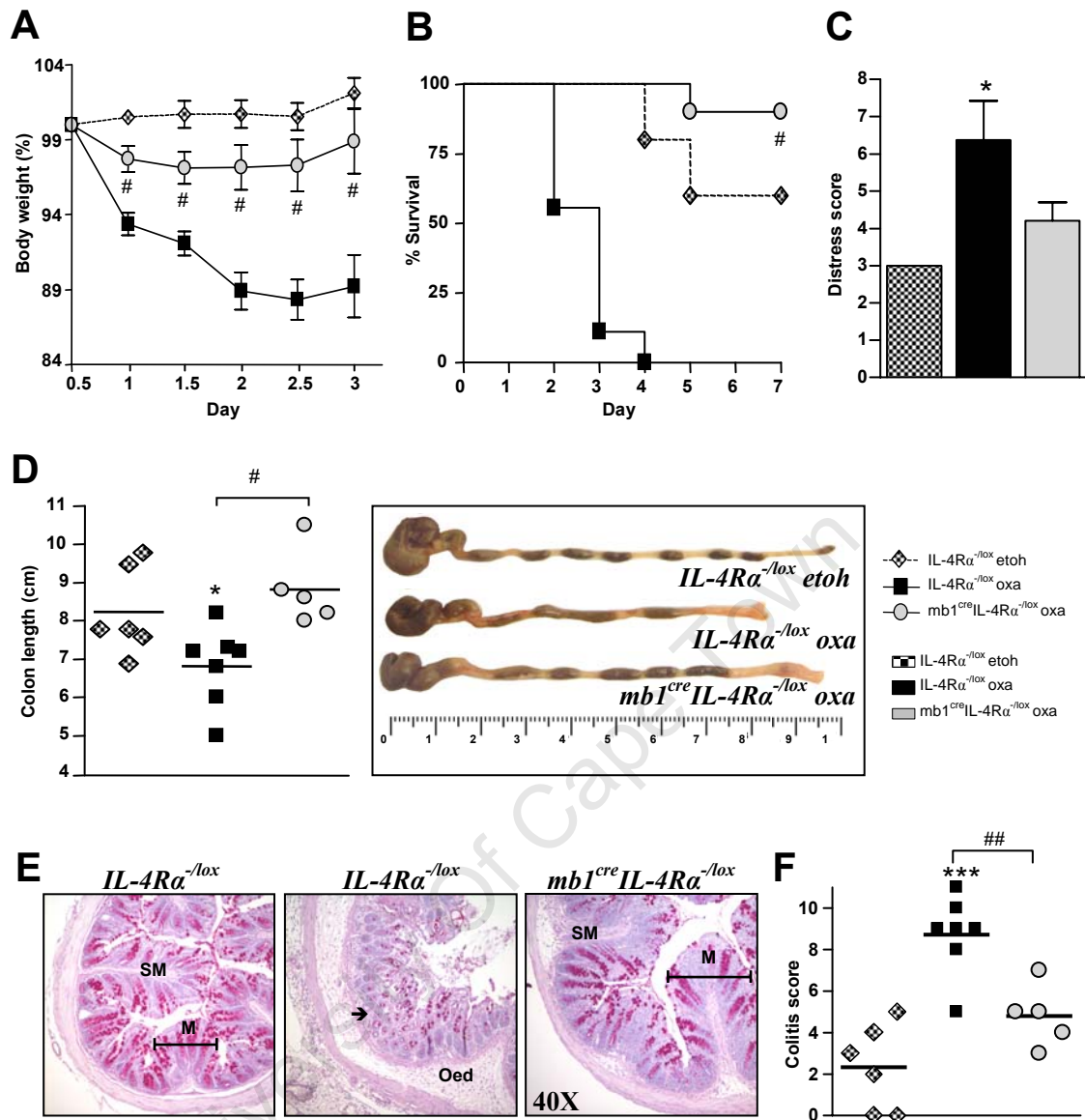
(D) Normal allergic response to ovalbumin. PAS stained histology sections showed mucus production by goblet cells (G) and cellular infiltration (I) in the lungs of ovalbumin sensitized IL-4R $\alpha^{-/-}$  and mb1<sup>cre</sup>IL-4R $\alpha^{-/-}$  mice. Data represents 3 independent experiments (n=4), mean values are  $\pm$  SEM and \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs. IL-4R $\alpha^{-/-}$ .

***B cell-specific IL-4R $\alpha$ -deficient mice are protected from oxazolone-induced colitis.***

Antibody-mediated hypersensitivity has long been considered a principal mediator of tissue damage in ulcerative colitis (UC) (Kett *et al.* 1987; MacDonald and Monteleone 2006). B cell-specific IL-4R $\alpha$ -deficient mice were generated using the Cre/loxP system with Cre-recombinase expression under control of the regulatory region for *mb1*. The *mb1* gene encodes the Ig- $\alpha$  signalling subunit of the B cell antigen receptor and therefore resulted in IL-4R $\alpha$  deletion specifically on B cells (Reth and Wienands 1997; Herbert *et al.* 2004; Hobeika *et al.* 2006). IL-4R $\alpha$ -deletion on B cells was functionally characterized in a mouse model of allergy induced by ovalbumin. Considering the lack of IgE and IgG1 antibody response in a Th2 driven allergy model in novel *mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>* mice, we used an oxazolone-induced model of Th2 colitis (Baird *et al.* 1998; Heller *et al.* 2002) to determine the role of IL-4 responsive B cells in UC.

Oxazolone-treated *mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>* mice were significantly protected from rapid wasting disease (Figure 4A) and death (Figure 4B) induced in IL-4R $\alpha$ <sup>-lox</sup> mice. Distress was scored according to; appearance, clinical signs, natural and provoked behaviour and was reduced in B cell-specific IL-4R $\alpha$ -deficient mice (Figure 4C). The macroscopic appearance and length of the colon was normal in *mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>* mice with less inflammation-induced colon shortening (Figure 4D). For oxazolone-induced colitis in IL-4R $\alpha$ <sup>-lox</sup> control mice, the histopathological picture was similar to that seen in human UC, described by oedema, infiltration of granulocytes, epithelial layer disruption and loss of mucus production (Figure 4E). Colitis scoring proved *mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>* mice to be protected from the immunopathology associated with Th2 colitis, compared with IL-4R $\alpha$ <sup>-lox</sup> mice (Figure 4F). Ethanol-only control mice showed no wasting disease and mild clinical and macroscopic symptoms of colonic inflammation.

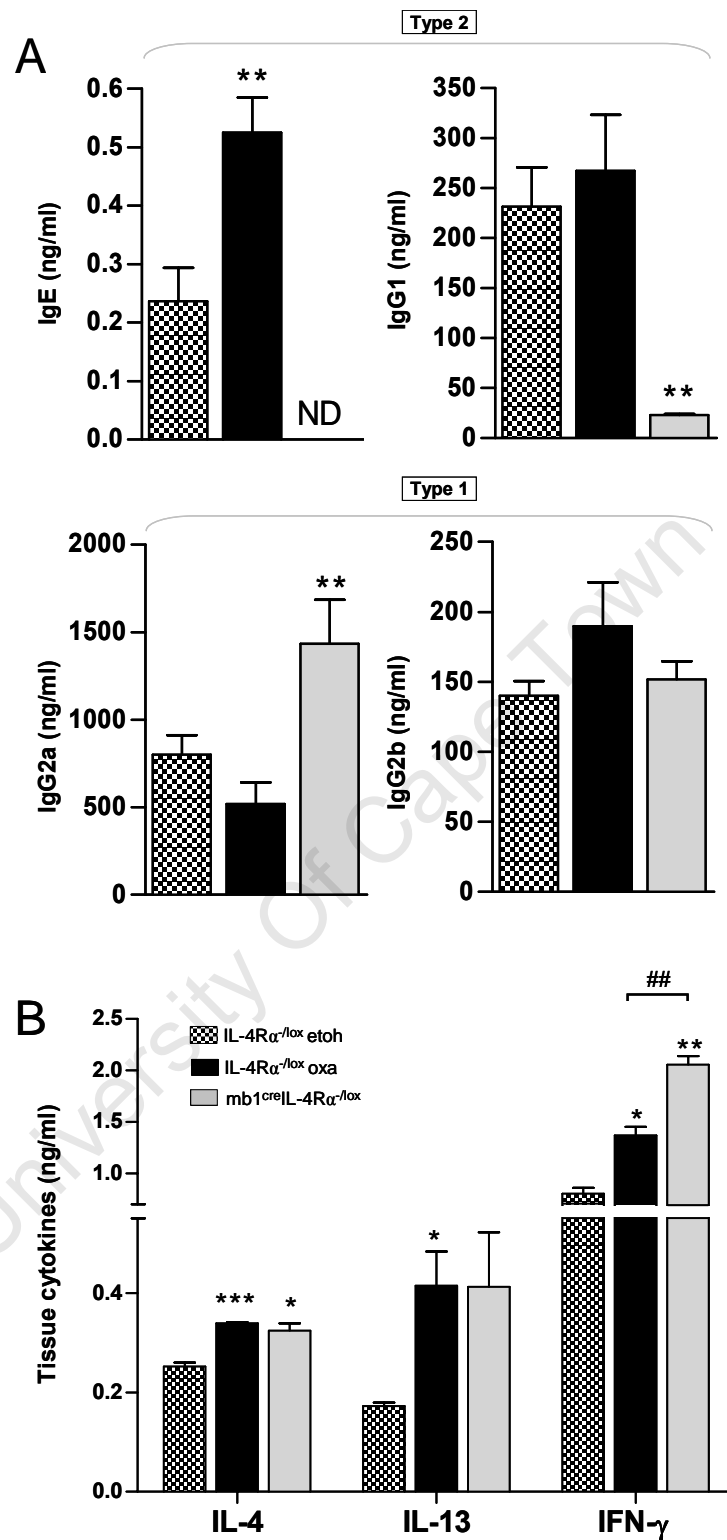
To address the impact of B cell-specific IL-4R $\alpha$ -deficiency on Th2 effector functions, we measured total antibody response in the serum and tissue cytokine response in the colon tissue. As seen in the ovalbumin allergy model, reduced IgE and IgG1 levels were detected (Figure 5A) despite a maintained Th2 cytokine response (Figure 5B). This suggests that B cells signalling via the IL-4R $\alpha$  to produce type 2 associated antibodies mediate oxazolone colitis. These data suggest that IL-4 responsive B cells and type 2 antibodies play an important role in the pathology caused by oxazolone-induced colitis.



**Figure 4: Mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice are protected from oxazolone-induced colitis.**

IL-4Rα<sup>-lox</sup> or mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> BALB/c mice were presensitized 7 days before intrarectal challenge with oxazolone or ethanol-only control.

(A) Wasting disease in oxazolone colitis. Weight loss was measured as a percentage starting weight for 3-7 days post-challenge. (B) Mortality was recorded with 10 mice /group. (C) Distress was measured and mice were scored on appearance, clinical signs, natural and provoked behaviour (D) Macroscopic changes of colons in oxazolone-treated mice. Colon shortening and inflammation limited to the distal half are symptoms associated with colitis. (E and F) Reduced oxazolone-mediated histopathology. (E) Colonic sections from IL-4Rα<sup>-lox</sup> or mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice administered oxazolone or ethanol only were stained with PAS. Representative photomicrographs (x40) are shown per group and presence of mononuclear cells (→), oedema (oed), SM =submucosa and M = mucosa are indicated (E). Semi-quantitative histopathological assessment of colitis activity was performed based on the presence of mononuclear cells, oedema, epithelial damage, loss of goblet cells and granulocyte infiltration (F). Data shown represents mean values ± SEM obtained from 2 independent experiments. Significance is compared with ethanol treated IL-4Rα<sup>-lox</sup> mice, \* = p < 0.05 and \*\*\* = p < 0.001 and IL-4Rα<sup>-lox</sup> vs. mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> was and # = p < 0.05 and ## = p < 0.01.



**Figure 5: Mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice have an impaired IgE response to oxazolone**

**A)** Impaired type 2 antibody response. Serum was collected from mice at day 3 post-challenge and total antibody levels were measured by ELISA.

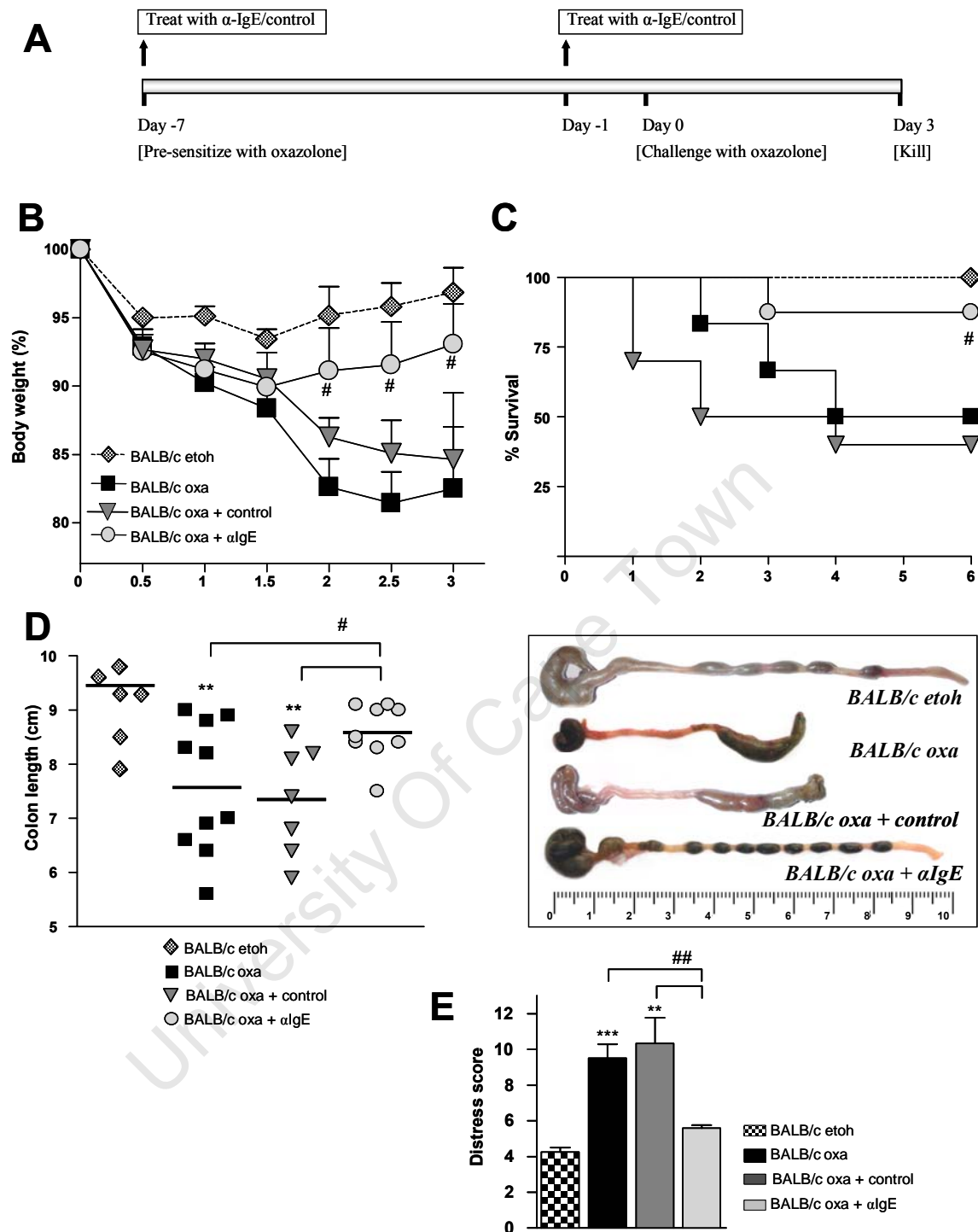
**(B)** Maintained Th2 cytokine response in the colon. Distal colon tissue was digested and prepared to measure cytokine levels in triplicate from pooled mice (n=4-8). Data represents 2 individual experiments with mean values  $\pm$  SEM and \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs. ethanol treated IL-4Rα<sup>-lox</sup> mice. IL-4Rα<sup>-lox</sup> vs. mb1<sup>cre</sup>IL-4Rα<sup>-lox</sup> was ## =  $p < 0.01$ . ND = not detected.

***Anti-IgE treatment protects BALB/c mice from oxazolone-induced colitis.***

As  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice were protected from colitis and presented with undetectable IgE and reduced IgG1 levels, we examined the inhibitory effects of anti-IgE or anti-Fc $\gamma$ RII/III (for IgG isotypes) treatment on oxazolone-induced colitis in BALB/c mice. Mice were treated with blocking antibodies the day before sensitization or challenge with oxazolone (Figure 6A). Mice treated with anti-IgE were significantly protected from rapid wasting disease (Figure 6B) and death (Figure 6C) compared to wild type and anti-rat IgG control BALB/c mice. Furthermore, the macroscopic appearance and length of the colon was normal in mice depleted of IgE with reduced inflammation-induced colon shortening (Figure 6D) and distress caused by the colitis (Figure 6E).

Figure 7A shows that anti-IgE treatment effectively depleted IgE but did not significantly alter IgG levels. As seen in  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice, IL-4 cytokine levels remained elevated despite the undetectable IgE. In contrast, IL-13 production was reduced in mice treated with anti-IgE (Figure 7B).

Photomicrographs showed IgE-depleted mice to be protected from the immunopathology associated with Th2 colitis, compared with wild type and control BALB/c mice (Figure 8A). This was confirmed by scoring the colitis according to disease severity (Figure 8B). The high numbers of mast cells found in the gastrointestinal tract and their potency to release pro-inflammatory mediators, highlight them to be involved in intestinal pathologies (Bischoff and Gebhardt 2006). An increase in mast cell numbers have been observed in patients during active ulcerative colitis (Bischoff *et al.* 1996). Mast cells are activated by crosslinking of IgE receptor, therefore to determine a role for mast cells in oxazolone colitis we quantified the number of mast cells observed in oxazolone-treated BALB/c mice and compared these numbers to mice treated with anti-IgE. Histological sections of the distal colon were stained with toluidine blue to visualize intestinal mast cells (Figure 8C). Quantification of mast cells showed a significant increase in wild type and anti-rat IgG control BALB/c mice treated with oxazolone (Figure 8D). However, mice treated with antibodies against IgE had mast cell numbers comparable to ethanol-only control mice. Together these data highlight a possible role for IgE (produced by IL-4 responsive B cells) in recruiting and activating mast cells, therefore mediating the pathology associated with oxazolone-induced colitis.

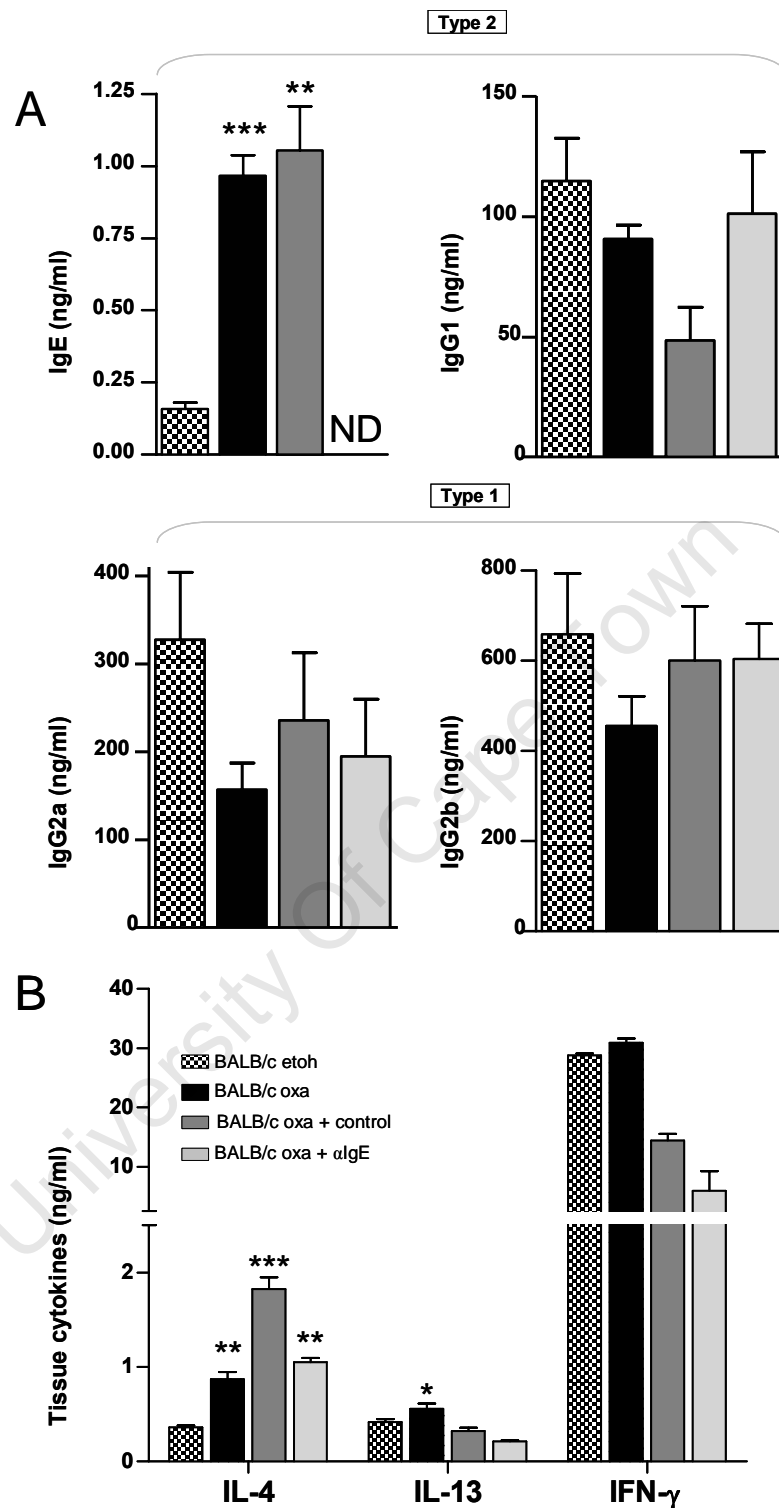


**Figure 6: Blocking IgE protects BALB/c mice from oxazolone-induced colitis.**

(A) BALB/c mice were treated with anti-IgE or control antibody one day before presensitized and again one day before intrarectal challenge with oxazolone or ethanol-only control. Mice were killed on day 3 post-challenge or day 6 for survival studies.

(B) Protection from wasting disease. Weight loss was measured as a percentage starting weight for 3 or 7 days post-challenge. (C) Significantly reduced mortality was recorded after anti-IgE treatment with 10 mice /group. (D) Protection from Macroscopic changes of colons in oxazolone-treated mice. Colon shortening and inflammation limited to the distal half of the colon were assessed.

(E) Distress was measured and mice were scored on appearance, clinical signs, natural and provoked behaviour (day 2). Data shown represents mean values  $\pm$  SEM obtained from 2 independent experiments from mice killed at day 3 unless stated otherwise. Significance is compared with ethanol treated BALB/c mice, \*\* = p < 0.01 and BALB/c oxa or anti-rat IgG control vs. BALB/c anti-IgE was # = p < 0.05 and ## = p < 0.01.

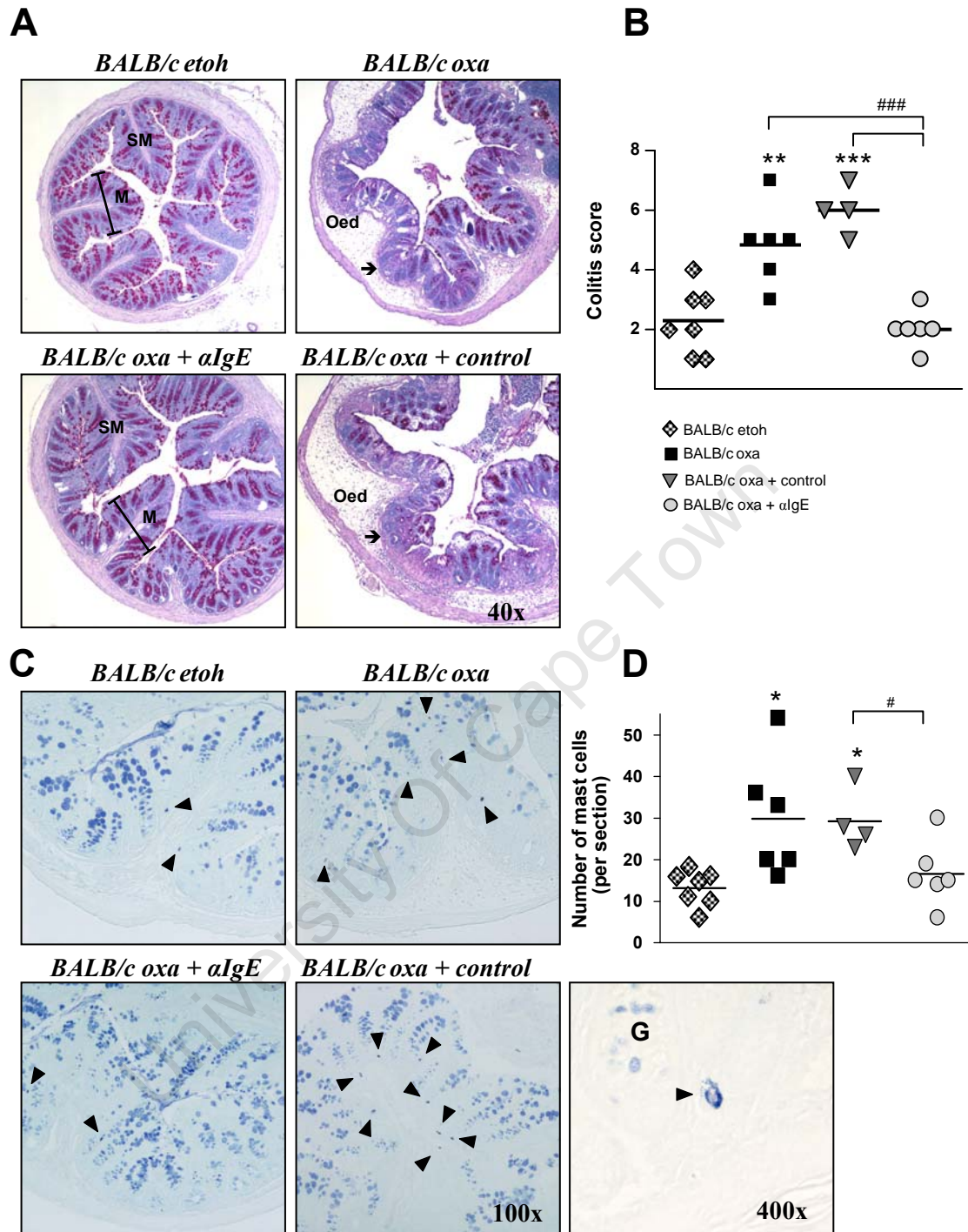


**Figure 7: BALB/c mice treated with anti-IgE maintain a Th2 cytokine response.**

**A)** Impaired IgE but not IgG1 antibody response. Serum was collected from mice at day 3 post-challenge and total antibody levels were measured by ELISA.

**(B)** Maintained IL-4 cytokine response in the colon. Colon tissue was digested and prepared to measure cytokine levels in triplicate from pooled mice (n=4-8). Data represents 2 individual experiments with mean values  $\pm$  SEM and \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs. ethanol treated BALB/c mice. ND = not detected.





**Figure 8: Depleting IgE reduces mast cell activation and oxazolone-induced colitis in BALB/c mice.**

IgE was blocked in BALB/c mice followed by sensitization and challenge with oxazolone or ethanol-only control. Mice were killed 3 days after challenge and colon sections were prepared to analyze oxazolone-induced inflammation or mast cell numbers. Representative photomicrographs are shown.

(A) Histological sections of colonic inflammation in IL-4Rα<sup>-lox</sup> mice administered oxazolone or ethanol only and treated with anti-IgE or control were stained with PAS (x40). The presence of mononuclear cells (➔), oedema (oed), submucosa (SM) and mucosa (M) are indicated.

(B) Semi-quantitative histopathological assessment of colitis activity was performed based on the presence of mononuclear cells, oedema, epithelial damage, loss of goblet cells and granulocyte infiltration. (C) Mast cells (▶) were stained with toluidine blue (x100 and x400) and quantified per section. Goblet cells (G) are indicated (D). Data shown represents mean values ± SEM obtained from 2 independent experiments. Significance is compared with ethanol treated BALB/c mice, \* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 and BALB/c vs. anti-IgE treated mice was # = p < 0.05 and ### = p < 0.001.

Ulcerative colitis has been associated with the production of IgG antibodies by plasma cells (MacDonald and Monteleone 2006). As  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice have reduced IgE and IgG1 levels we wanted to determine if IgG antibodies played a role in oxazolone-induced colitis. Therefore, we performed depletion studies using anti-Fc $\gamma$ RII/III treated mice. Blocking Fc $\gamma$ RII/III signalling had no effect on the outcome of disease in oxazolone-induced colitis with treated mice showing symptoms comparable to wild type or anti-rat IgG control BALB/c mice. Table 3 summarizes the results showing a significant weight loss comparable to oxazolone-treated wild type BALB/c mice.

**Table 3: Disease activity in oxazolone treated BALB/c mice treated with anti-Fc $\gamma$ RII/III.**

Groups	Body weight <sup>a</sup>	Distress Score <sup>b</sup>	Colon length	Colitis Score
<b>IL-4R<math>\alpha^{-/lox}</math> etoh</b>	95.19 $\pm$ 2.1	3.61 $\pm$ 0.6	8.84 $\pm$ 0.3	4.20 $\pm$ 0.5
<b>IL-4R<math>\alpha^{-/lox}</math> oxa</b>	82.61 $\pm$ 2.1 **	12.81 $\pm$ 1.5 ***	6.57 $\pm$ 0.4 **	9.67 $\pm$ 0.3 ***
<b>IL-4R<math>\alpha^{-/lox}</math> oxa + anti-Fc<math>\gamma</math>RII/III</b>	87.44 $\pm$ 1.4 *	10.00 $\pm$ 1.3 **	7.00 $\pm$ 0.7 *	8.25 $\pm$ 0.8 **

<sup>a</sup> Body weight is shown as percentage starting weight 2 days post-challenge.

<sup>b</sup> Distress is scored according to appearance, clinical signs, natural and provoked behaviour (day 2).

<sup>c</sup> Colon length is measured in centimetres.

\* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs BALB/c etoh control.

Furthermore, BALB/c mice treated with antibodies against Fc $\gamma$ RII/III were significantly more distressed than ethanol-only control mice and showed colon shortening associated with inflammation comparable to oxazolone-treated BALB/c mice. Histology sections of the distal colon showed signs of colitis with an increase in the presence of mononuclear cells, goblet cell depletion, granulocytes (specifically neutrophils) infiltration and oedema. The levels of IgG antibodies in the serum were comparable to oxazolone-treated BALB/c mice because anti-Fc $\gamma$ RII/III blocks the receptor of IgG antibodies. Therefore, these antibodies were produced in response to oxazolone but were unable to signal. These data suggest that IgG antibodies do not play a role in mediating oxazolone colitis but that IgE antibodies induce disease pathology.

## DISCUSSION

IL-4 and IL-13 regulate the function of many cell-types and are important in the generation of immunity in human diseases. Both IL-4 and IL-13 share a common signalling pathway through IL-4R $\alpha$  (Nelms *et al.* 1999). By using Cre/loxP recombination to delete the IL-4R $\alpha$  in cell-specific transgenic mice, the role of IL-4 and/or IL-13 responsive B cells could be investigated. IL-13 has no functional receptor on T cells and mouse B cells were reported to be unresponsive to IL-13, however evidence suggests mouse IL-13 to enhance antibody production *in vivo*, therefore acting directly on B cells (Zurawski and de Vries 1994; Lai and Mosmann 1999; Mohrs *et al.* 2000). By disrupting IL-4R $\alpha$  specifically on B cells, the detrimental role of both IL-4 and IL-13 signalling on these cells could be investigated in a mouse model of ulcerative colitis. We described the generation, characterization and functional analysis of mice lacking the IL-4R $\alpha$  chain selectively on B lymphocytes (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice). In an allergic asthma model, mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice had an impaired type 2 antibody response to ovalbumin confirming functional disruption of IL-4R $\alpha$ . However, these mice remained susceptible to the lung pathology associated with allergy and maintained a Th2 cytokine response. Mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice also had an impaired type 2 antibody response, with no-IgE detectable in oxazolone-induced colitis and were significantly protected from disease onset. IgE was highlighted as a mediator of oxazolone-induced colitis as anti-IgE treatment protected BALB/c mice from developing disease.

The novel B cell-specific IL-4R $\alpha$ -deficient mouse strain (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>) was generated using the Cre/loxP recombination system in BALB/c ES cells as previously demonstrated for macrophage/neutrophil- (Herbert *et al.* 2004), T cell- (Radwanska *et al.* 2007; Dewals *et al.* 2009) and smooth muscle cell-specific (Horsnell *et al.* 2007) mice. The efficiency of B cell-specific IL-4R $\alpha$  deletion was increased by mating IL-4R $\alpha$ <sup>-/-</sup> with transgenic mb1<sup>cre</sup> mice, and then crossing them with IL-4R $\alpha$ <sup>lox/lox</sup> mice, thereby reducing the loxP substrate for Cre-recombinase by 50%. PCR genotyping confirmed transgene-bearing, hemizygous mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice and FACS analysis showed efficient B cell-specific IL-4R $\alpha$  disruption on spleen and bone marrow CD19<sup>+</sup> and B220<sup>+</sup> cells. The normal expression of IL-4R $\alpha$  on T cell subpopulations, NK cells and macrophages confirmed the cellular specificity of disruption with functional analysis showing normal IL-4-driven proliferation and differentiation in CD4<sup>+</sup> lymphocytes. Naïve wild type mice demonstrate low but detectable levels of non-specific antibodies. IgE was undetectable in

mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> naïve mice with very low levels of IgG1 suggesting a disruption of B cell function in response to IL-4.

To functionally characterize the disruption of IL-4R $\alpha$  on B cells in mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice, an *in vivo* model of allergic asthma in response to ovalbumin was induced. Comparison of these mice to IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/-</sup> controls showed that impaired IL-4R $\alpha$  signalling in B cells had no effect on the major symptoms of allergic asthma but that mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice had significantly reduced levels of Th2-associated antibodies (IgE and IgG1). The increase in type 1 antibodies (IgG2a and IgG2b) and trend to higher IFN- $\gamma$ , suggests that although these mice develop allergic asthma they show a shift towards a Th1 response. The maintained levels of Th2 cytokines, especially IL-13 would explain the ovalbumin-induced allergic response (Grunig *et al.* 1998; Wills-Karp *et al.* 1998). Furthermore, spleen, mediastinal lymph node and lung CD19<sup>+</sup> cells showed impaired IL-4 dependent surface expression of the low affinity receptor for IgE (CD23), supporting data that proved IL-4R $\alpha$  to be functionally disrupted specifically on B cells (Kijimoto-Ochiai 2002).

Antibody-mediated hypersensitivity is a strong candidate for mediating tissue damage in UC. Even appendectomy has been shown to protect against the development of UC (Rutgeerts *et al.* 1994). It is suggested that the appendix plays a role in primary B cell development in rabbits (Pospisil and Mage 1998) and by removing it, the colon auto-reactive or cross-reactive specificities would also be removed. This highlights a role for B cells in UC, however the mechanisms of their involvement and responsiveness to IL-4/IL-13 is poorly understood. Colitis was induced in IL-4R $\alpha$ <sup>-/lox</sup> BALB/c mice by intrarectal administration of oxazolone subsequent to skin sensitization. A chronic inflammation limited to the distal colon was obtained which proved to be similar to human UC in that it was relatively superficial, showed ulceration and had mixed inflammatory infiltrate of lymphocytes and granulocytes. As 50% ethanol alone has also been shown to induce a mild colitis in the colon, mice treated with ethanol-only were included as controls for all experiments (Andrade *et al.* 2003).

Our research showed that mice with B cells unable to respond to IL-4/IL-13 were significantly protected from oxazolone-induced colitis. These mice maintained a Th2 polarized response, demonstrated by increased IL-4 and IL-13 levels but had an abrogated type 2 antibody response with no detectable IgE. Previous studies have

highlighted the role of IL-13 producing NKT cells in oxazolone colitis (Heller *et al.* 2002) but  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice have IL-13 and IL-4 levels comparable to  $IL-4R\alpha^{-/lox}$  mice. Therefore, it is interesting that despite non-B cells maintaining their ability to respond to IL-13, the lack of type 2 antibodies was sufficient to confer protection from colitis in  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice. Given the heterogeneity of ulcerative colitis it is possible that blocking various signalling pathways at different stages of disease could equally protect from disease onset.

Experimental models of anaphylaxis show both IgE-dependent and independent mechanisms, with the former inducing IgE cross-linking of FcεRI in mast cells (Coyle *et al.* 1996; Strait *et al.* 2002; Nieuwenhuizen *et al.* 2007). In a previous study, blocking IgE in mice sensitized with ovalbumin had no effect on the outcome of disease suggesting an IgE-independent anaphylaxis and supporting our data in which  $mb1^{cre}IL-4R\alpha^{-/lox}$  mice have abrogated IgE but still present with an allergic response (Nieuwenhuizen *et al.* 2007). In colitis, blocking IgE has been shown to reduce most of the clinical signs, such as weight loss, colon shortening, rectal bleeding and diarrhoea using the DSS-induced mouse model (Kang *et al.* 2006). DSS has been shown to induce both acute and chronic colitis in mice resembling UC with the acute phase being an innate, T and B cell independent response (Okayasu *et al.* 1990; Dieleman *et al.* 1994). Chronic DSS-induced colitis is characterized by focal epithelial regeneration and both Th1 and Th2 cytokine profiles (Dieleman *et al.* 1998). As oxazolone-induced colitis is commonly used to study the contribution of Th2-dependent immune responses to intestinal inflammation, we wanted to determine if blocking IgE would protect mice treated with oxazolone, confirming an IgE-dependent mechanism for oxazolone-induced colitis. It should be mentioned that oxazolone at low doses can also induce a mixed Th1/Th2-dependent colitis; therefore we sensitized BALB/c mice 7 days prior to challenge (Wirtz and Neurath 2007).

Indeed our studies showed that blocking IgE significantly protected BALB/c mice against oxazolone-induced colitis with a corresponding reduction in mast cell numbers. This effect was IgE-dependent as blocking the FcγRII/III had no effect on the outcome of disease. In mice, the high affinity FcεRI receptor for IgE is limited to basophils and mast cells with mast cells being the major IgE-effectors whose function is to mediate cellular degranulation and release various inflammatory mediators (Kinet 1999). In support of our data, blocking IgE in mice treated with DSS reduced the expression of

mast cell-mediated inflammatory factors, indicating that intestinal mast cell activation through FcεRI plays a role in the pathogenesis of UC (Kang *et al.* 2006). Furthermore, a recent study showing that oral antigen-induced intestinal and systemic anaphylaxis depends on IgE and mast cells and requires IL-9/IL-9R signalling (Osterfeld *et al.* 2010). This supports our observations and strengthens the importance of IgE highlighting a role for mast cells in regulating gut inflammation in UC. To determine if mast cells are indeed the cause of oxazolone-induced colitis further studies on mast cells will be required. Such studies include, measurement of mouse mast cell protease-1 (MMCP-1) in the serum or mast cell depletion studies using anti-*c-kit* antibodies (McDermott *et al.* 2003).

In conclusion, our studies demonstrate that IL-4Rα expression on B cells plays a crucial role in mediating oxazolone-induced colitis. This colitis is IgE-dependent as blocking IgE confers protection from disease, while blocking the receptor for IgG antibodies has no affect on disease outcome. We also suggest a role for mast cells with increased numbers in susceptible oxazolone-treated BALB/c mice. These numbers are reduced by blocking IgE production and protecting mice from colitis.

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## **2.3 Experimental Study 3**

**An essential role for IL-4 responsive CD4<sup>+</sup> T cells in  
oxazolone-induced colitis**

### ABSTRACT

Effector responses induced by polarized T helper 2 (Th2) cells drive the pathology associated with oxazolone-induced colitis in BALB/c mice. The Th2 cytokines IL-4 and IL-13 are known to mediate the immune response in the oxazolone mouse model of ulcerative colitis. However, the CD4<sup>+</sup> T cell specific requirements of IL-4R $\alpha$  signalling are unclear. The purpose of this study was to determine the mechanism of oxazolone-induced colitis in gene-targeted mice with a deletion of the IL-4R $\alpha$  chain specifically on CD4<sup>+</sup> T lymphocytes (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>). Expression of the IL-4R $\alpha$  on CD4<sup>+</sup> T cells was critical for colitis induction as Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were protected from disease pathology shown by the amelioration of disease parameters including weight loss, survival, macroscopic inflammation, colitis shortening and histopathology. This protection was associated with reduced levels of IL-13 production. The importance of IL-4 responsive CD4<sup>+</sup> T cells in mediating oxazolone colitis was confirmed by adoptively transferring CD4<sup>+</sup>/DX5<sup>-</sup> non-NK T cells into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice to revert protection back to a susceptible phenotype in these mice. Together these data suggest that IL-4 responsive CD4<sup>+</sup> T cells are crucial in mediating oxazolone-induced colitis driven by increased levels of IL-13 production.

## INTRODUCTION

T cells have been linked to ulcerative colitis by the effectiveness of T cell-depletion therapy (Sawada *et al.* 2005) and ciclosporin treatment which reduces immune functioning of effector T cells (Lichtiger *et al.* 1994). Mouse models of Inflammatory Bowel Disease (IBD) have used T cell depletion and disruption of cytokine or cytokine receptor genes to show a disrupted T cell-dependent regulatory system. (Mombaerts *et al.* 1993; Morrissey *et al.* 1993; Sadlack *et al.* 1993; Strober and Ehrhardt 1993; Powrie 1995; Suzuki *et al.* 1995; Takahashi *et al.* 1997). Lamina propria CD4<sup>+</sup> T cells reacting to enteric flora are important effector cells causing mucosal inflammation in many experimental IBD models (Elson *et al.* 1995). Furthermore, studies of IBD in which CD4<sup>+</sup> T cells are depleted *in vivo*, shows that inflammation is ameliorated (Simpson *et al.* 1995; Okamoto *et al.* 1999). Together these IBD models show that subpopulations of CD4<sup>+</sup> T cells and their cytokines play a crucial role in the development of intestinal immune disorders.

Hapten-induced mouse models of colitis are useful tools in understanding the mechanisms of mucosal inflammation. While trinitrobenzene sulphonic acid (TNBS)-colitis is a Th1-mediated inflammation resembling human Crohn's disease (CD), oxazolone-colitis is a Th2-mediated inflammation resembling human ulcerative colitis (UC) (Morris *et al.* 1989; Yamada *et al.* 1992; Boirivant *et al.* 1998). Initial studies using a short-lived model of oxazolone colitis demonstrated that CD4<sup>+</sup> T cells produce large amounts of IL-4 and IL-5 and that blocking IL-4 prevented colitis (Boirivant *et al.* 1998). Subsequently, using a prolonged oxazolone colitis model, initial IL-4 production gave way to increasing levels of IL-13. By blocking IL-13 signalling with the soluble IL-13R $\alpha$ 2-Fc, mice were protected from oxazolone-induced colitis. Furthermore, NK T cells producing IL-13 were highlighted as mediators of disease (Heller *et al.* 2002; Bouma and Strober 2003; Heller *et al.* 2005). The authors proposed that APCs or epithelial cells present antigen to NK T cells through CD1 and that NK T cells then produce IL-13. NK T cells can therefore have direct cytotoxicity against epithelial cells or IL-13 mediates disease by inducing epithelial cell apoptosis. However, NK T cells in this study also produced increased levels of IL-4 in response to  $\alpha$ GalCer, a synthetic glycolipid that activates most invariant NK T cells (Kawano *et al.* 1997). Therefore, it is also possible that IL-4 plays a role in driving the initial inflammatory response to oxazolone and that early IL-4 production by CD4<sup>+</sup> cells initiates IL-13 production by NK T cells. To

investigate the role of IL-4 signalling on T cells during oxazolone-induced colitis we used mice deficient in the IL-4R $\alpha$  specifically on CD4<sup>+</sup> T cells (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>).

Previous studies in our lab have shown various roles for IL-4 responsive T cells in disease. Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice are protected from OVA-induced anaphylaxis despite the maintenance of a Th2 response (Nieuwenhuizen *et al.* 2007). These mice also survive acute schistosomiasis; where Th2 responses are essential for host survival (Leeto *et al.* 2006). Furthermore, T cell responses to IL-4 are responsible for susceptibility to *L. major* infection (Radwanska *et al.* 2007) and are the cause of hypersensitivity to *N. brasiliensis* (Mearns *et al.* 2008). These studies highlight the possibility of a role of IL-4 responsive CD4<sup>+</sup> T cells in mediating a Th2 model of colitis.

In this study, we addressed the role of CD4<sup>+</sup> T cell IL-4R $\alpha$  expression in UC. This was achieved by treating previously characterised Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice with oxazolone and monitoring disease outcome. Our results demonstrate that Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were significantly protected from the pathology associated with oxazolone colitis, indicating a role for IL-4 responsive CD4<sup>+</sup> T cells in mediating disease. To confirm that these cells do indeed drive pathology, we adoptively transferred IL-4 responsive CD4<sup>+</sup> T cells from IL-4R $\alpha$ <sup>-/lox</sup> littermate controls into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice and were able to revert protection back to a susceptible phenotype. As a subpopulation of NK T cells are also CD4 positive (Kronenberg and Gapin 2002), we confirmed the disease pathology to be mediated by CD4<sup>+</sup> non-DX5<sup>+</sup> NK T cells, by transferring CD4<sup>+</sup>/DX5<sup>-</sup> sorted cells back into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice and inducing pathology. These data suggest a potential role for IL-4 responsive CD4<sup>+</sup> non-NK T cells in mediating oxazolone-induced inflammation.

## MATERIALS AND METHODS

### *Mice.*

The generation and characterization of CD4<sup>+</sup> T cell-specific IL-4R $\alpha$ -deficient (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>) mice was previously described (Radwanska *et al.* 2007). Briefly as described in chapter 2, Lck<sup>cre</sup> mice on a BALB/c background were crossed with IL-4R $\alpha$ <sup>-/-</sup> mice (Mohrs *et al.* 1999) and IL-4R $\alpha$ <sup>lox/lox</sup> mice (Herbert *et al.* 2004) to generate Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice. Male mice (6-8 weeks old) were genotyped by PCR to confirm CD4<sup>+</sup> T cell-specific disruption of IL-4R $\alpha$  and hemizygous IL-4R $\alpha$ <sup>-/lox</sup> littermates were used as control groups in oxazolone colitis experiments. All mice were housed in specific pathogen-free conditions at the University of Cape Town, South Africa and experiments were approved by the University's Animal Ethics Committee.

### *Oxazolone-induced colitis and adoptive transfer of CD4<sup>+</sup> or CD4<sup>+</sup>/DX5<sup>-</sup> cells.*

Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> and IL-4R $\alpha$ <sup>-/lox</sup> littermate control mice were treated with oxazolone as described in chapters 2 and 3. Anesthetized mice were sensitized with 3% oxazolone in 100% ethanol (150 $\mu$ l) followed 7 days later by intrarectal administration of 1% oxazolone in 50% ethanol (150 $\mu$ l). Mice were monitored daily for weight loss and killed 3 days post-challenge for immunopathological analyses or 7 days post-challenge for survival studies. For adoptive transfer studies, lymphocytes from IL-4R $\alpha$ <sup>-/lox</sup> mice were FACS sorted for CD4<sup>+</sup> (clone: GK1.5) or CD4<sup>+</sup>/DX5<sup>-</sup> (clone: DX5) cells (>98% purity). Biotin-labelled antibodies were detected by streptavidin-APC and anti-Fc $\gamma$ RII/III (clone: 2.4G2) was used to block non-antigen-specific binding of immunoglobulins to Fc $\gamma$ RII and Fc $\gamma$ RIII receptors. Antibodies were from BD Pharmingen (San Diego, CA). Mice received 1x10<sup>6</sup> sorted cells 24hr prior to sensitization with oxazolone (see Appendix A for additional reagents for all experiments).

### *Disease activity index (DAI).*

Oxazolone-treated IL-4R $\alpha$ <sup>-/lox</sup> control mice develop rapid-onset colitis marked by weight loss and distress as described in chapters 2 and 3. Essentially, disease progression was monitored by weight loss as a percentage starting weight and distress scored at day 2 post-challenge (see Table 1 of Appendix A) (Wolfensohn and Lloyd 1998). Survival studies were performed with 10 mice/ group and the same criteria were monitored as mentioned above. Colon length was measured from the anus to the caecum and recorded as an indication of inflammation.

***Histological assessment of colitis.***

Distal colon sections taken 1cm from the anus were fixed in ice cold 4% phosphate-buffered formalin to prevent tissue autolysis. Histology sections were processed and stained with haematoxylin and eosin for inflammatory cells or PAS reagent for mucus producing goblet cells. Semi-quantitative histopathological grading of oxazolone-induced colitis was adjusted from a previous study, (Iijima *et al.* 2004) with 5 criteria (presence of mononuclear cells, reduced goblet cells, epithelial injury, granulocyte infiltration and oedema). Each were scored from 0-3 with an additive score of between 0 (no colitis) and 15 (maximal colitis activity).

***Isolation and restimulation of splenocytes.***

As described in chapters 2 and 3, individual or pooled spleens from oxazolone treated mice were collected in complete IMDM supplemented with 10% FCS, penicillin, streptomycin, 1mM sodium pyruvate, 50 $\mu$ M  $\beta$ 2-ME and NEAA. Viable single cells (depleted of RBCs) were diluted to 1x10<sup>6</sup> cells/ml and cultured in complete IMDM with anti-CD3 (10 $\mu$ g/ml) for 48h at 37°C and 5% CO<sub>2</sub>. Supernatants were collected and stored at -80°C.

***Cytokine and total IgE detection by ELISA.***

Sandwich ELISAs were performed to determine cytokine levels in cell supernatants and total serum IgE levels as described in chapters 2 and 3. Essentially, microtitre plates were coated with purified anti-IL-4, anti-IFN- $\gamma$ , anti-IL-13 or anti-IgE antibodies, diluted in PBS. Serially diluted recombinant IL-4, IFN- $\gamma$ , IL-13 or IgE standards, cell supernatants or serum was added. Biotinylated anti-mouse secondary antibodies for IL-4, IFN- $\gamma$ , IL-13 or IgE were added followed by HRP labelled streptavidin for detection. Subsequently, plates were incubated with TMB Peroxidase Substrate, the colour reaction stopped with 2M H<sub>2</sub>SO<sub>4</sub> and absorption measured at 450nm. ELISA wash buffer was used to wash plates 4x between each step.

***Statistical analysis.***

Values are given as mean  $\pm$  SEM, and significant differences were determined using unpaired two-tailed students *t* test or one-way ANOVA using a Bonferroni post-test (GraphPad Prism). Values of *p* <0.05 were considered significant.

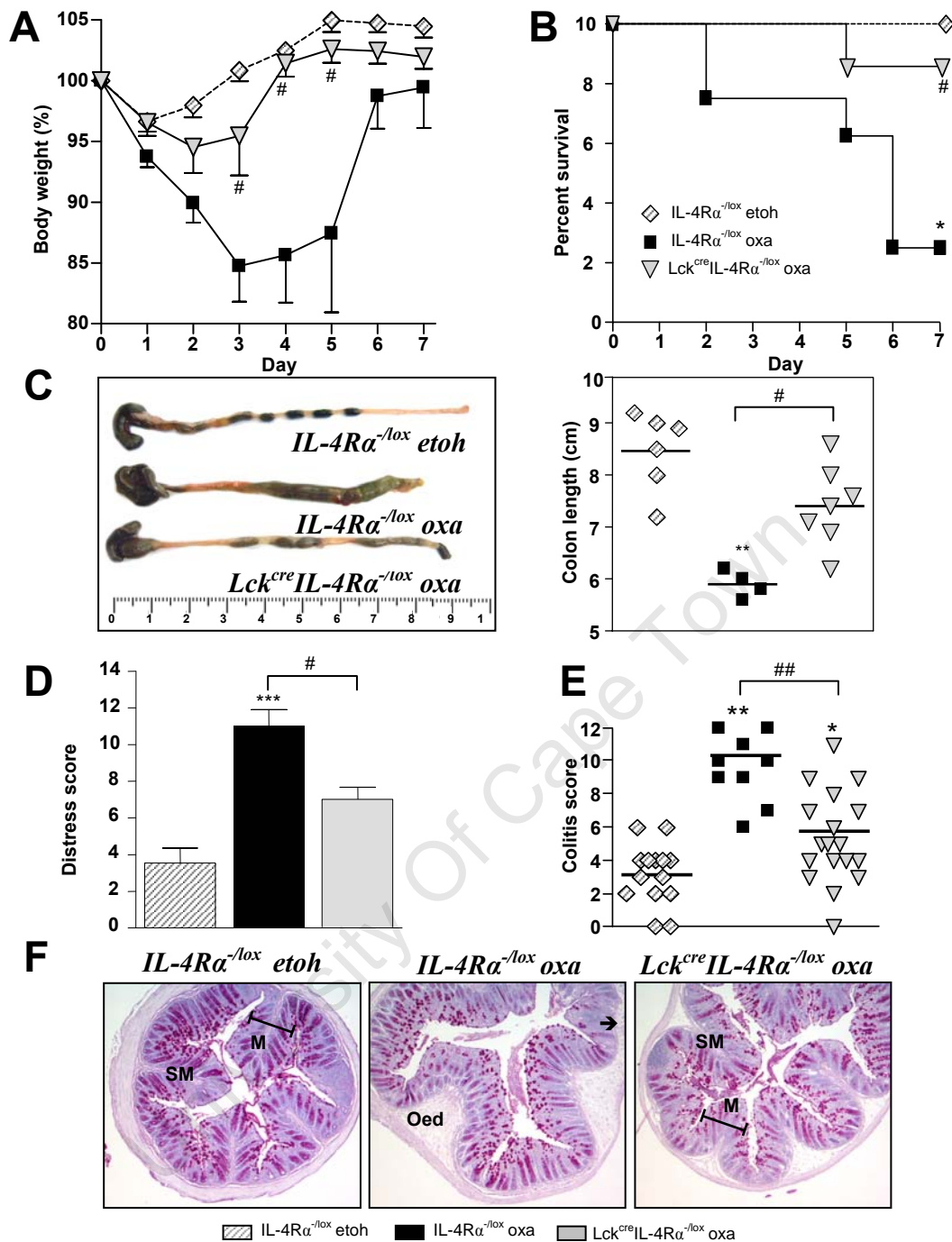


## RESULTS

### ***CD4<sup>+</sup> T cell-specific IL-4R $\alpha$ -deficient mice are protected from oxazolone colitis.***

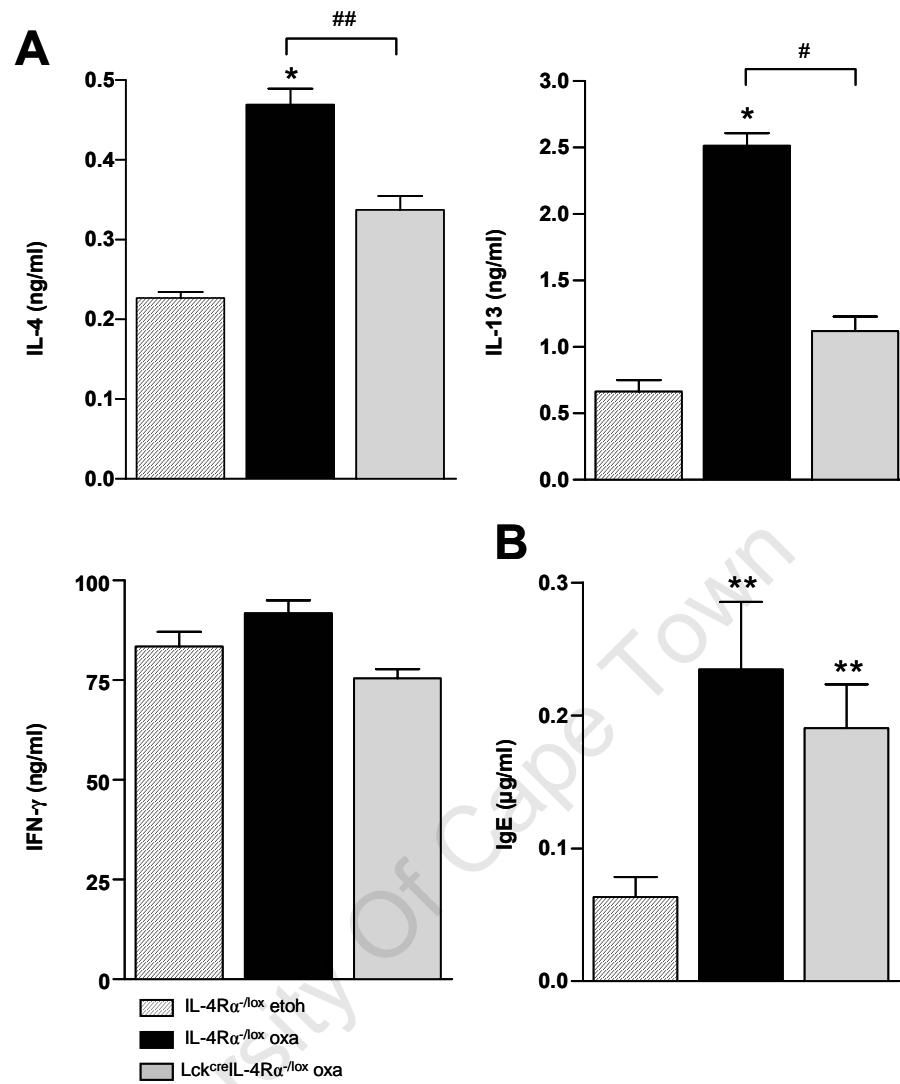
As murine T lymphocytes do not have a functional IL-13 receptor (Zurawski and de Vries, 1994) specifically deleting the IL-4R $\alpha$  from CD4<sup>+</sup> T cells will determine a role for IL-4 responsive T cells during oxazolone-induced colitis. Therefore we treated mice deficient in IL-4R $\alpha$  on CD4<sup>+</sup> T cells (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>) to determine the role of IL-4 responsive CD4<sup>+</sup> T cells in oxazolone-induced colitis.

Previously characterised Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice have complete disruption of IL-4R $\alpha$  on CD4<sup>+</sup> T cells with variable deletion efficiency on CD8<sup>+</sup>,  $\gamma\delta$ <sup>+</sup> and NK T cells (Radwanska *et al.* 2007). Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were protected from oxazolone-induced colitis shown by comparable weight loss (Figure 1A) and survival (Figure 1B) to ethanol-treated control IL-4R $\alpha$ <sup>-/lox</sup> mice. The macroscopic appearance and length of the colon was normal in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice with less inflammation-induced colon shortening (Figure 1C). Distress was scored according to appearance, clinical signs, natural and provoked behaviour, and was significantly reduced in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice (Figure 1D). Although Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice showed mild symptoms of pathology, with some inflammatory cell infiltration and oedema, the colitis score in these mice remained significantly lower than oxazolone-treated IL-4R $\alpha$ <sup>-/lox</sup> control mice (Figure 1E and 1F). The protection of Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice from oxazolone-induced colitis was associated with significantly reduced levels of IL-4 and IL-13 cytokine production and normal levels of IFN- $\gamma$  (Figure 2A). As previously shown in response to *N. brasiliensis*, Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice maintained production of IgE despite reduced levels of IL-4 (Figure 2B). These data demonstrate that IL-4 responsive CD4<sup>+</sup> T cells mediate oxazolone-induced colitis.



**Figure 1: Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice are protected from oxazolone-induced colitis.**

CD4<sup>+</sup> T cell-specific IL-4Rα-deficient BALB/c mice were pre-sensitized with 3% oxazolone (oxa) in 100% ethanol before intrarectal challenge with 1% oxa in 50% ethanol (Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> oxa). Littermate control mice were administered ethanol only (IL-4Rα<sup>-lox</sup> etoh). Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice were protected from rapid onset of colitis induced by oxazolone shown by: **(A)** Significantly reduced weight loss as percentage starting weight and **(B)** prolonged survival compared with IL-4Rα<sup>-lox</sup> controls treated with oxazolone (day 7). **(C)** Normal colon appearance in macroscopic pictures with normal colon length, indicating reduced inflammation. **(D)** Significantly less distress, scored according to severity of symptoms with a maximum score of 16 at day 2 post-challenge. **(E)** Significantly reduced colitis score from pooled experiments. **(F)** Reduced or no oxazolone-induced inflammation shown in PAS-stained sections of the distal colon, while IL-4Rα<sup>-lox</sup> mice show oedema (oed), reduced mucous production and inflammatory infiltrates (→). SM = submucosa and M = mucosa. All data represents mice killed 3 days post-challenge unless indicated. Data represents 3 individual experiments (n=4-8 mice) with mean values ± SEM and \* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs. IL-4Rα<sup>-lox</sup> etoh controls and # = p < 0.05 and ## = p < 0.01 IL-4Rα<sup>-lox</sup> vs. Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> oxa.



**Figure 2: Impaired Th2 response to oxazolone in Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice.**

Systemic T lymphocyte responses were assessed from spleen cells of oxazolone treated CD4<sup>+</sup> T cell-specific IL-4Rα-deficient mice and compared to oxazolone treated or control IL-4Rα<sup>-/-</sup> littermates.

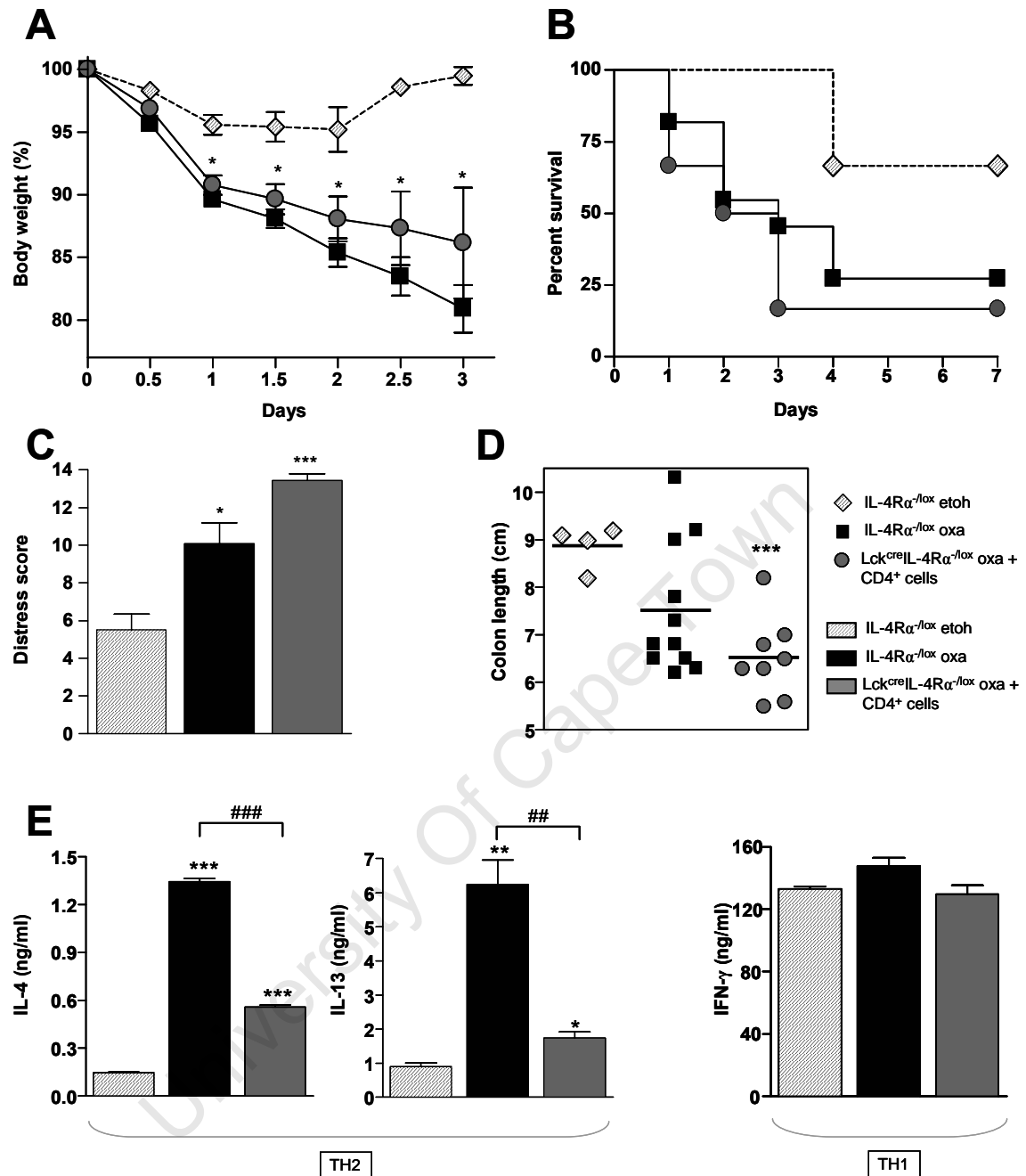
**(A)** Cytokine production from restimulated splenocytes was analysed by ELISA showing significantly reduced IL-4 and IL-13 production with little or no effect on IFN-γ levels. **(B)** Despite reduced levels of IL-4 and IL-13, Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice were able to produce IgE antibodies in response to oxazolone treatment. Total IgE levels were determined by ELISA from the serum of experimental mice 3 days post-challenge (10 days post-sensitization). Data represents 2 individual experiments (n =4-8), with mean ± SEM and \* = p <0.05 and \*\* = p <0.01 vs. IL-4Rα<sup>-/-</sup> etoh mice and # = p <0.05 and ## = p <0.01 IL-4Rα<sup>-/-</sup> vs. Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> oxa mice.

***IL-4 responsive CD4<sup>+</sup> T cells mediate oxazolone-induced colitis.***

We showed that BALB/c mice with CD4<sup>+</sup> T cells unable to respond to IL-4 (Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup>) were protected from oxazolone-induced colitis suggesting that IL-4 responsive CD4<sup>+</sup> T cells mediate colitis. To confirm this data we therefore adoptively transferred IL-4 responsive CD4<sup>+</sup> T cells from IL-4Rα<sup>-/lox</sup> into Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice to determine whether we could revert these mice back to the disease phenotype seen in IL-4Rα<sup>-/lox</sup> mice.

Indeed, Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice receiving IL-4 responsive CD4<sup>+</sup> T cells developed oxazolone-induced colitis. This was shown with weight loss comparable to oxazolone-treated IL-4Rα<sup>-/lox</sup> mice (Figure 3A) and a similar mortality rate (Figure 3B). Ethanol treatment alone has also been shown to induce a mild form of colitis in the colon. This was evident in the mortality rate of IL-4Rα<sup>-/lox</sup> mice treated with ethanol-only as a negative control (Figure 3B). Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice receiving IL-4 responsive CD4<sup>+</sup> T cells were significantly distressed compared to ethanol-only control mice (Figure 3C) with colon shortening comparable to oxazolone-treated IL-4Rα<sup>-/lox</sup> mice (Figure 3D). IL-4 and IL-13 production by spleen cells was significantly increased in Lck<sup>cre</sup>IL-4Rα<sup>-/lox</sup> mice which received CD4<sup>+</sup> T cells compared with ethanol-only control mice, while IFN-γ production remained unchanged (Figure 3E). Although the level of these Th2 cytokines were lower than oxazolone treated IL-4Rα<sup>-/lox</sup> mice, the small amounts produced were sufficient to drive the pathology associated with oxazolone colitis.

These data confirm the importance of CD4<sup>+</sup> T cells in mediating oxazolone-induced colitis. However, it does not rule out the possibility that CD4<sup>+</sup> NK T cells are in fact driving the pathology associated with oxazolone colitis or that the action of transferring cells alone is causing some form of pathology. Therefore we planned to transfer CD4<sup>+</sup>/DX5<sup>-</sup> T cells to all mouse groups to determine if IL-4 responsive NK T cells are involved in driving oxazolone colitis.



**Figure 3: IL-4 responsive CD4<sup>+</sup> T cells mediate oxazolone-induced colitis in Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  mice.**

CD4<sup>+</sup> T cells from IL-4R $\alpha^{-/-}$  mice were sorted by FACS (>98% purity) and adoptively transferred into Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  mice. Control IL-4R $\alpha^{-/-}$  mice administered ethanol-only or oxazolone also received CD4<sup>+</sup> cells. Oxazolone-treated mice were treated as described above with pre-sensitization and challenge steps. CD4<sup>+</sup> T cells were able to mediate the onset of oxazolone-induced colitis in Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  mice shown by; (A) Significant weight loss as percentage starting weight. (B) Increased mortality comparable to oxazolone-treated IL-4R $\alpha^{-/-}$  control mice (day 7). (C) Significant distress compared with IL-4R $\alpha^{-/-}$  ethanol controls, scored according to severity of symptoms with a maximum score of 16 at day 2 post-challenge. (D) Reduced colon length indicating inflammation.

(E) Systemic T lymphocyte responses were assessed from restimulated splenocytes of oxazolone treated mice and showed IL-4 and IL-13 production to be lower than oxazolone-treated control mice but maintained a polarized Th2 response (E). No significant difference was detected in IFN- $\gamma$  production.

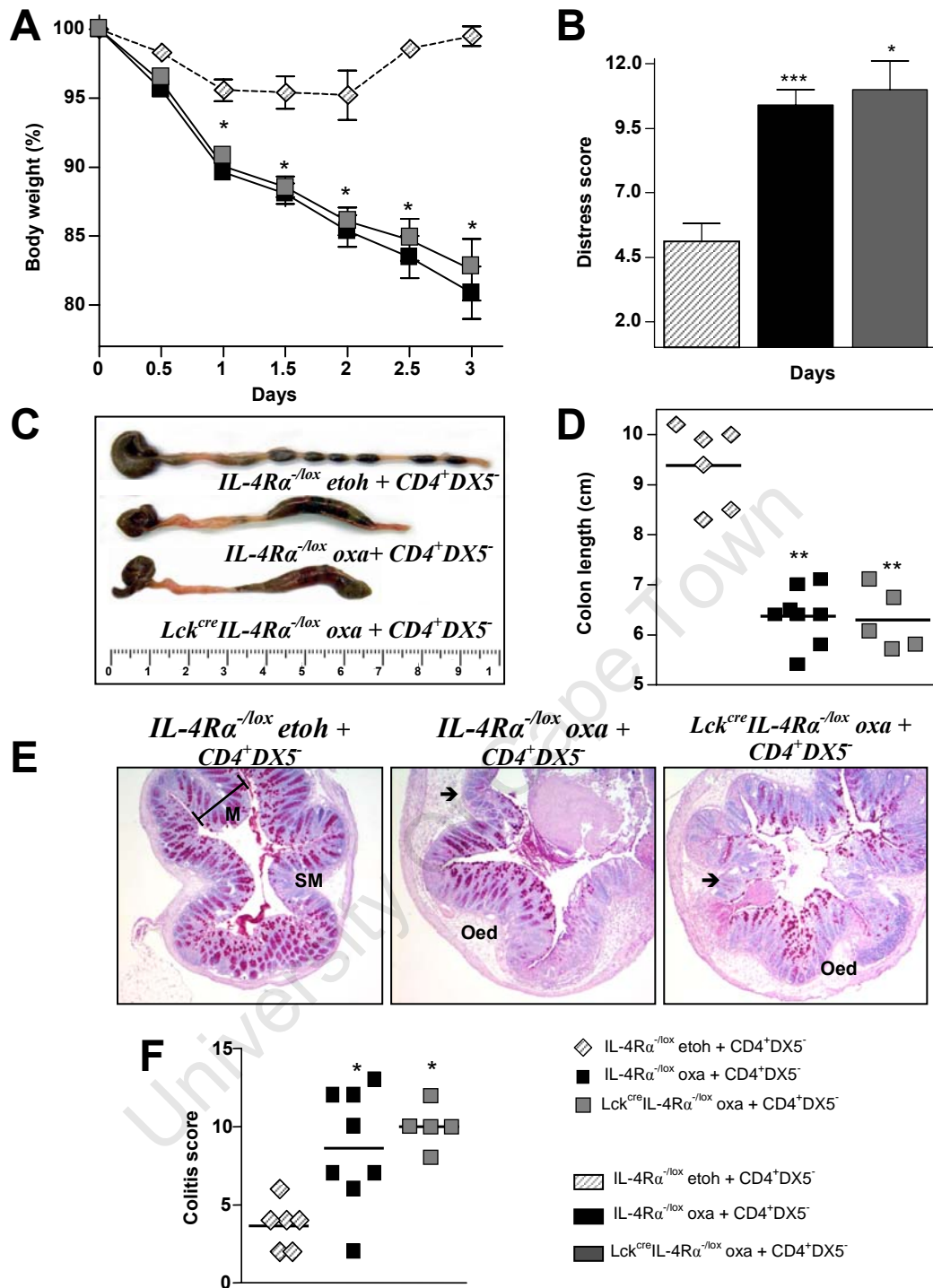
All data represents mice killed 3 days post-challenge unless indicated. Data represents 2 individual experiments (n=4-8 mice) with mean values  $\pm$  SEM and \* =  $p < 0.05$ , \*\* =  $p < 0.01$  and \*\*\* =  $p < 0.001$  vs. IL-4R $\alpha^{-/-}$  etoh controls and ## =  $p < 0.01$  and ### =  $p < 0.001$ , IL-4R $\alpha^{-/-}$  vs. Lck<sup>cre</sup>IL-4R $\alpha^{-/-}$  oxa.

***IL-4 responsive NK T cells are not essential in oxazolone-induced colitis.***

Previous studies have suggested that experimental and human ulcerative colitis is mediated directly by NK T cells, described as CD4<sup>+</sup> T cells bearing NK markers, in the lamina propria (Heller *et al.* 2002; Fuss and Strober 2008). These NK T cells could be activated to secrete IL-13, which may bring about changes in the epithelial barrier function or have direct cytotoxic effects on epithelial cells. In oxazolone-induced colitis IL-4 is produced initially and then superseded by increased IL-13 production (Heller *et al.* 2002). Our data suggests a role for IL-4 responsive CD4<sup>+</sup> T cells in mediating oxazolone colitis; however we could not eliminate the possibility that these are CD4<sup>+</sup> NK T cells. To determine if oxazolone colitis could be induced in mice lacking IL-4 responsiveness on CD4<sup>+</sup> non-NK T cells, we adoptively transferred CD4<sup>+</sup>DX5<sup>-</sup> T cells expressing IL-4R $\alpha$  into protected Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice and IL-4R $\alpha$ <sup>-lox</sup> mice treated with or without oxazolone as controls.

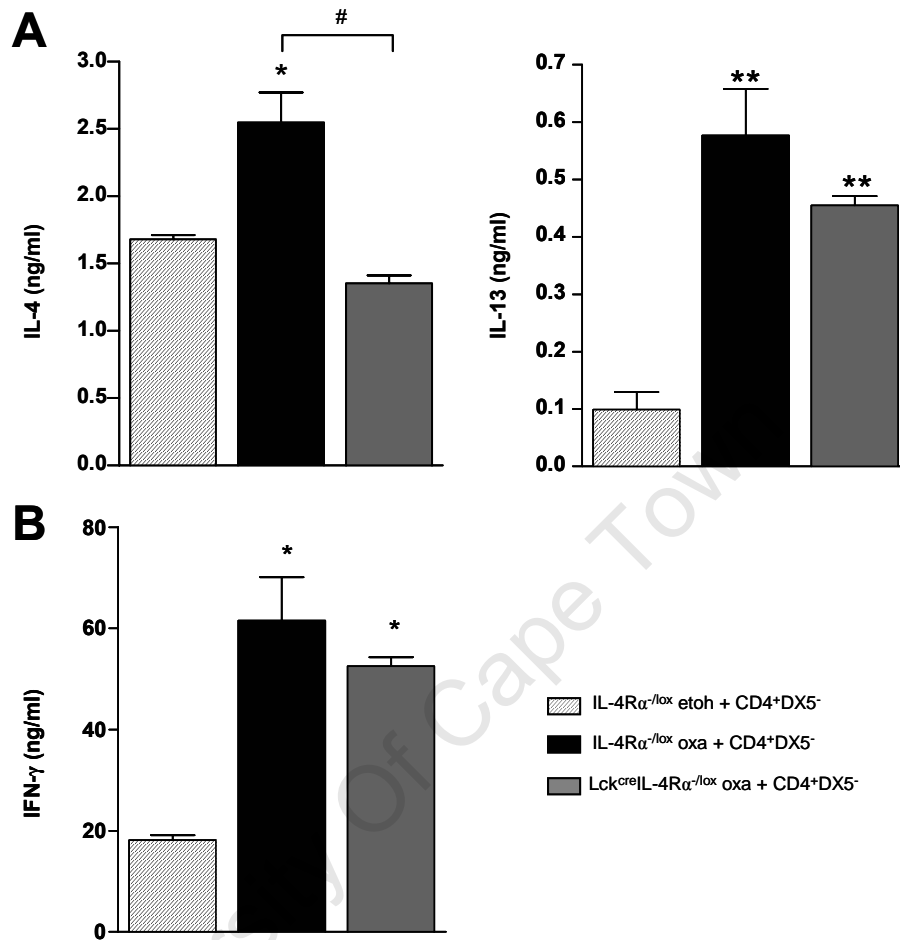
Our data show that CD4<sup>+</sup> non-NK T cells mediate oxazolone-induced colitis. This is shown by significant weight loss presented as a percentage starting weight (Figure 4A) and significant distress (Figure 4B). Macroscopic examination three days post-challenge showed severe colitis limited to the distal half of the colon (Figure 4C) with inflammation associated colon shortening compared with ethanol-only control mice (Figure 4D). Microscopic examination of the distal colon showed superficial inflammation characterized by epithelial cell loss, patchy ulceration and pronounced depletion of mucous producing-goblet cells (Figure 4E), confirmed by semi-quantitative histopathological assessment (Figure 4F). Although the systemic levels of IL-4 production remained low, IL-13 was increased and associated with oxazolone-induced pathology in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup> mice treated with IL-4 responsive CD4<sup>+</sup> non-NK T cells (Figure 5A). As IFN- $\gamma$  production has been shown to be variable in the oxazolone colitis model depending on dose and time point of detection, the elevated levels in both oxazolone treated groups is not unexpected (Figure 5B).

Together these data strongly suggests that IL-4 responsive CD4<sup>+</sup> non-NK T cells are involved in mediating oxazolone-induced colitis. Although T lymphocytes are thought not to have a functional IL-13 receptor, the possibility that IL-13 signals via the newly described IL-13R $\alpha$ 2 to cause pathology should not be excluded.



**Figure 4: IL-4 responsive DX5<sup>+</sup> NK T cells are not essential in oxazolone-induced colitis.**

As CD4 is expressed on some NK T cells we sorted CD4<sup>+</sup>/DX5<sup>-</sup> T cells by FACS (>98% purity) to exclude the possibility of NK T cells mediating oxazolone-induced colitis. Therefore, CD4<sup>+</sup>/DX5<sup>-</sup> T cells were adoptively transferred into Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice. Control IL-4Rα<sup>-lox</sup> mice administered ethanol-only or oxazolone also received CD4<sup>+</sup>/DX5<sup>-</sup> T cells. CD4<sup>+</sup>/DX5<sup>-</sup> T cells maintained their ability to mediate the onset of oxazolone-induced colitis in Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice shown by; (A) Significant weight loss as percentage starting weight. (B) Significant distress compared with IL-4Rα<sup>-lox</sup> ethanol controls, at day 2 post-challenge. (C) Macroscopic appearance of inflammation limited to the distal colon. (D) Reduced colon length. (E) Inflammation shown in PAS-stained sections of the distal colon, oedema (oed), inflammatory infiltrates (→), SM=submucosa and M=mucosa and confirmed by semi-quantitative histopathological assessment (F). All data represents mice killed 3 days post-challenge unless indicated. Data represents 1 individual experiment (n=5-8 mice) with mean values ± SEM and \* = p < 0.05, \*\* = p < 0.01 and \*\*\* = p < 0.001 vs. IL-4Rα<sup>-lox</sup> etoh controls.



**Figure 5: IL-4 responsive CD4<sup>+</sup>/DX5<sup>-</sup> T cells induce IL-13 production in oxazolone-treated Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice.**

Systemic T lymphocyte responses were assessed from spleen cells of oxazolone-treated Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> mice which received CD4<sup>+</sup>/DX5<sup>-</sup> T cells by adoptive transfer.

(A) Systemic cytokine production from restimulated splenocytes was analysed by ELISA showing significantly reduced IL-4, but maintained compared to IL-4Rα<sup>-/-</sup> littermate control mice treated with oxazolone. (B) IFN-γ production was elevated in both mouse groups treated with oxazolone compared to ethanol-only control mice. Data represents 1 individual experiment (n = 5-8) of mice killed on day 3, with mean ± SEM and \* = p < 0.05 and \*\* = p < 0.01 vs. IL-4Rα<sup>-/-</sup> etoh mice and # = p < 0.05 IL-4Rα<sup>-/-</sup> vs. Lck<sup>cre</sup>IL-4Rα<sup>-/-</sup> oxa mice.



## DISCUSSION

In the present study we describe a BALB/c model of ulcerative colitis mediated by IL-4 responsive CD4<sup>+</sup> non-NK T cells. Experimental models of IBD allow us to elucidate cellular and molecular mechanisms of disease pathology. Previous studies showed direct involvement of CD4<sup>+</sup> T cells in colitis by adoptively transferring CD45 RB<sup>high</sup> CD4<sup>+</sup> T cells into skid mice to induce intestinal inflammation (Powrie *et al.* 1994; Powrie 1995). In this model, the transfer of DX5<sup>+</sup> NKT cells reduced established colitis suggesting a regulatory role for these cells in a chronic type 1-mediated colitis (Hornung *et al.* 2006). Furthermore, CD4<sup>+</sup> TCR $\alpha$  $\beta$ <sup>+</sup> T cells mediate the development of UC in TCR $\alpha$ <sup>-/-</sup> mice (Mombaerts *et al.* 1993; Viney *et al.* 1994; Takahashi *et al.* 1997). While IL-4 mediates multiple effects on murine T cells, these cells do not respond to IL-13 (Zurawski and de Vries 1994). Therefore, the availability of CD4<sup>+</sup> T cell-specific IL-4R $\alpha$ -deficient mice (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>) allows investigation of the role of IL-4 signalling specifically on CD4<sup>+</sup> T cells while maintaining IL-4/IL-13-mediated functions on non-CD4<sup>+</sup> T cells.

Previously characterized and published Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice (Nieuwenhuizen *et al.* 2007; Radwanska *et al.* 2007; Dewals *et al.* 2009) were sensitized and challenged with oxazolone. Our data shows these mice to be significantly protected from oxazolone-induced colitis shown by the amelioration of disease parameters including weight loss, survival, macroscopic inflammation, colitis shortening and histopathology. Abrogated disease pathology in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice was associated with a decrease in IL-13 production by spleen cells. Although IL-13 is thought to be the major player in oxazolone colitis, IL-4 is also able to disrupt epithelial cell tight junctions (Ceponis *et al.* 2000). The data in chapter 2 of this thesis show that IL-4<sup>-/-</sup> BALB/c mice are partially protected from colitis. These mice have significantly increased levels of IL-13 after oxazolone treatment, whereas Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice have IL-13 levels comparable to ethanol-only control mice. Therefore it remains likely that IL-13 is the primary mediator of colitis with the low levels of IL-13 in Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice responsible for attenuated disease development. These data also suggest that IL-4 signalling through non-CD4<sup>+</sup> T cells may regulate IL-13 production.

To confirm the role of IL-4 responsive CD4<sup>+</sup> T cells in oxazolone colitis we adoptively transferred these cells back into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice and were able to drive oxazolone-induced colitis. These data demonstrate the importance of IL-4 responsive CD4<sup>+</sup> T cells in mediating colitis but could not distinguish the effects of classical CD4<sup>+</sup> non-NK T

cells and CD4<sup>+</sup> NK T cells. Therefore, we attempted to deplete NK T cells by FACS sorting for CD4<sup>+</sup>/CD49b<sup>-</sup> (DX5) lymphocytes. There is controversy about whether CD49b (DX5) suitably identifies subpopulations of NK T cells, as only a minority of DX5<sup>+</sup> cells are CD1d-dependent (Pellicci *et al.* 2005). However, most CD1d-dependent NK T cells express DX5 (Pellicci *et al.* 2005) therefore we depleted NK T cells by removing CD4<sup>+</sup>/DX5<sup>+</sup> T cells. Adoptive transfer of IL-4 responsive CD4<sup>+</sup> non-DX5<sup>+</sup> NK T cells into Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice resulted in oxazolone-induced colitis, suggesting an important role for these cells in disease pathology. Furthermore, Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice which received these cells produced significantly increased levels of IL-13 highlighting the possible importance of IL-13 in disease progression.

Although IL-13 production by NK T cells has been shown to mediate oxazolone-induced colitis, our data show that IL-4 responsive CD4<sup>+</sup> non-NK T cells are also important in mediating disease. Whether this occurs before or after NK T cell activation and IL-13 production remains to be defined.

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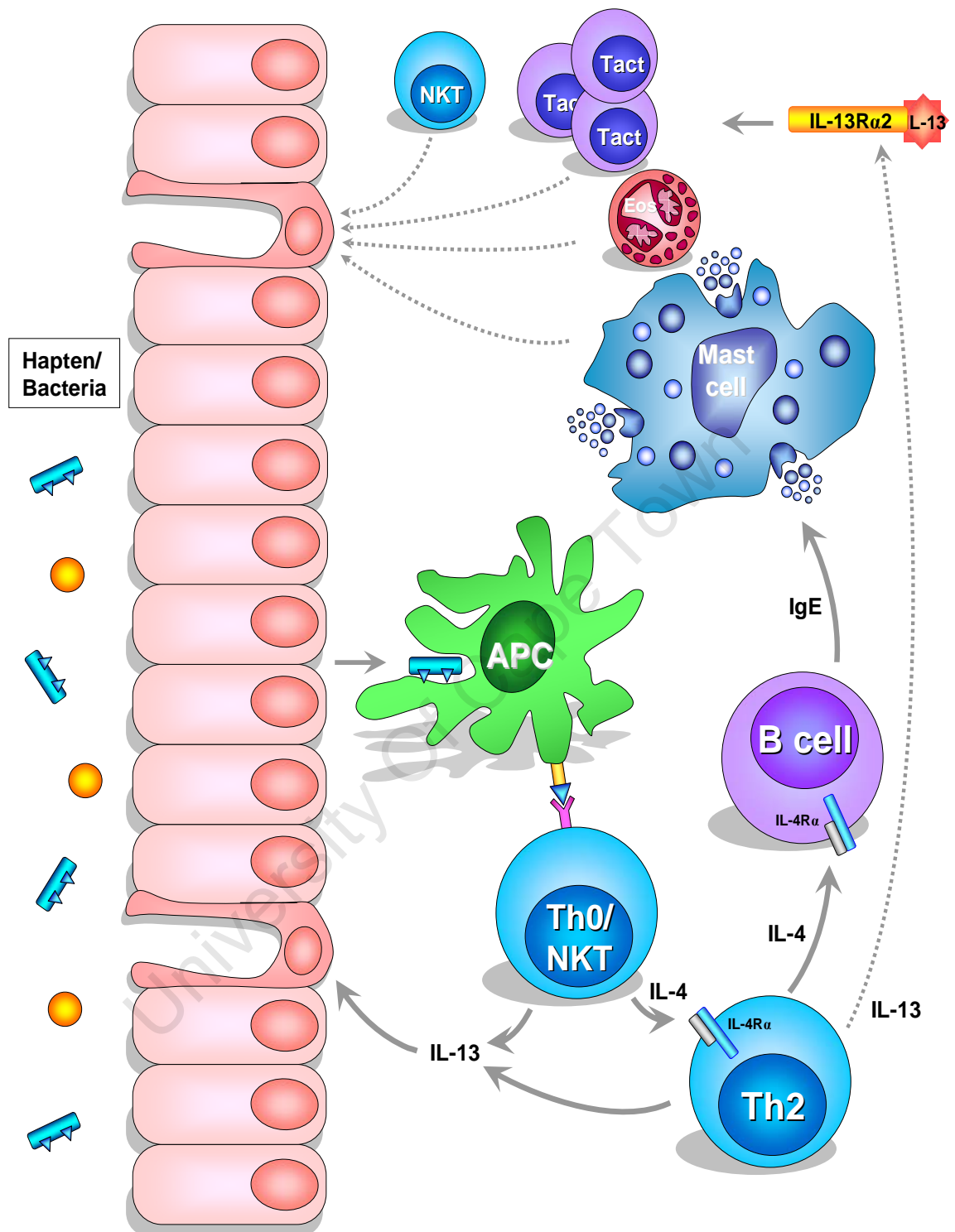
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## **Chapter 3: General Discussion and Perspectives**

Animal models have played an important role in understanding the underlying mechanisms of pathogenesis in ulcerative colitis. The main focus of this project was to investigate the role of Th2 cytokines and the receptors through which they signal in a mouse model of UC induced by oxazolone. By using a combination of IL-4, IL-13 and IL-4R $\alpha$ -deficient mice, the first study suggests a possible role for IL-13 signalling independently of the IL-4R $\alpha$  in mediating colonic inflammation. In the second study, we characterized a novel genetic mouse model in which IL-4R $\alpha$  was specifically deleted on B cells (mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>). By using these mice in the oxazolone-induced colitis model we could demonstrate that IL-4 responsive B cells are involved in driving the disease. As mb1<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice failed to launch an IgE response to oxazolone treatment we investigated whether IgE is critical for colitis pathology. Indeed, depleting IgE from BALB/c mice significantly protected them from the onset of colitis with reduced mast cell numbers in the distal colon. In the third study, we used a previously characterized T cell-specific IL-4R $\alpha$ -deficient mouse (Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup>). Our data showed that IL-4 responsive CD4<sup>+</sup> T cells were critical for colitis induction as Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice were protected from disease pathology. We confirmed the importance of IL-4 responsive CD4<sup>+</sup> T cells but not CD4<sup>+</sup>DX5<sup>+</sup> NK T cells by adoptively transferring IL-4R $\alpha$  expressing CD4<sup>+</sup>DX5<sup>-</sup> T cells into Lck<sup>cre</sup>IL-4R $\alpha$ <sup>-/lox</sup> mice and managed to reverse protection back to a susceptible phenotype.

Combining our data with previous studies, a hypothetical model can be drawn on the possible mechanisms involved in the pathogenesis of oxazolone colitis (Figure 1). Essentially, commensal bacteria products stimulate epithelium or the mucosal immune system by penetrating the mucosal barrier. Antigen presenting cells (APCs) or direct stimulation from epithelial cells activates NK T cells to drive a Th2/type 2 response. Alternatively, APCs stimulate Th0 cells to drive a Th2 response, subsequently activating NK T cells as a downstream effect. Together these NK T cells or IL-4 dependent Th2 cells drive the pathology associated with oxazolone colitis. NK T and Th2 cells both produce IL-13 which is toxic to epithelial cells. Furthermore, IL-13 signalling via the IL-13R $\alpha$ 2 induces an inflammatory response. Lastly, Th2 cells produce IL-4 which stimulates B cells to drive inflammation in an IgE-dependent manner, increasing mast cell numbers.



**Figure 1: Hypothetical model of the pathogenesis of oxazolone colitis.**

Commensal bacteria products stimulate epithelium or the mucosal immune system by penetration of the mucosal barrier. Antigen presenting cells (APCs) or direct stimulation from epithelial cells activate NK T cells to drive a Th2/type 2 response. Alternatively activated Th0 cells can drive a Th2 response, subsequently activating NK T cells. NK T and Th2 cells produce IL-13 which is toxic to epithelial cells. IL-13 signalling via the IL-13Rα2 induces an inflammatory response. Lastly, Th2 cells produce IL-4 which stimulates B cells to drive inflammation in an IgE-dependent manner, increasing mast cell numbers.



In study one, our data confirms previous studies showing the importance of IL-4 and IL-13 production in oxazolone-induced colitis. However we show for the first time that by blocking the receptor through which these cytokines signal (IL-4R $\alpha$ ) disease was exacerbated. Furthermore, we demonstrate that IL-13 mediates colitis in IL-4R $\alpha^{-/-}$  and wild type mice, as disrupting IL-13 but not IL-4 production in BALB/c and IL-4R $\alpha^{-/-}$  mice rescues them from the pathology associated with oxazolone colitis. This suggests a role for IL-4R $\alpha$ -independent signalling of IL-13 to induce colitis. Although IL-13 clearly drives pathology, the source of this IL-13 is yet to be defined. As shown in our data, many cell types express the IL-13R $\alpha$ 2. However, further studies are required to determine if cells expressing IL-13R $\alpha$ 2 are responsible for inducing the pathology of oxazolone colitis. As epithelial cells have been shown to express IL-13R $\alpha$ 2 (Donaldson *et al.* 1998), studies isolating these cells and measuring the effect of IL-13 on IL-13R $\alpha$ 2 expression would be of interest. Our results also show the downregulated expression of IL-13R $\alpha$ 2 on neutrophils in oxazolone treated mice. IL-13 has been shown to activate neutrophils and it would be interesting to determine if these effects are related to the IL-13R $\alpha$ 2 (Girard *et al.* 1996).

In study two, we demonstrate that mice deficient in IL-4R $\alpha$  specifically on B cells (mb1<sup>cre</sup>IL-4R $\alpha^{-/lox}$ ) are significantly protected from oxazolone-induced colitis. These mice had reduced levels of IgE and IgG1. Furthermore, blocking IgE protects BALB/c mice from oxazolone colitis with reduced mast cell numbers. Therefore, IgE production by IL-4 responsive B cells induces inflammation in response to oxazolone. Although we were able to detect increased mast cell numbers in the distal colon of oxazolone-treated mice, further analysis would be useful in determining mast cell activation. Once degranulation occurs mast cells are not seen in histology sections, but mast cell-derived mediators such as tryptase and TNF- $\alpha$  can be detected in tissue samples. To effectively determine the importance of mast cells in oxazolone colitis, mast cell depletion studies would be advantageous. Furthermore, staining for IgE in the distal colon, could determine the localization of IgE in the inflamed tissue. Interestingly, although IL-4R $\alpha^{-/-}$  mice have no detectable levels of IgE (Experimental study 1, Figure 6), they show exacerbated disease. It is possible that the regulatory role of non-T and B cells in response to IL-4 or IL-13 may be more crucial in protecting mice from disease pathology.

In study three, we addressed the role of IL-4 responsive CD4<sup>+</sup> T cells in oxazolone colitis. Our results demonstrate the significant protection of Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice from the pathology associated with oxazolone colitis, indicating a role for IL-4 responsive CD4<sup>+</sup> T cells in mediating disease. To confirm that these cells do indeed drive pathology, we adoptively transferred IL-4Rα expressing CD4<sup>+</sup> T cells from IL-4Rα<sup>-lox</sup> littermate controls into Lck<sup>cre</sup>IL-4Rα<sup>-lox</sup> mice and were able to revert protection back to a susceptible phenotype. As a subpopulation of NK T cells are also CD4 positive, we confirmed the disease pathology to be mediated by IL-4Rα expressing CD4<sup>+</sup>DX5<sup>-</sup> T cells by removing CD4<sup>+</sup>DX5<sup>+</sup> (NK T cells), before adoptive transfer. These data suggest a potential role for IL-4 responsive CD4<sup>+</sup> but not NK T cells in mediating oxazolone-induced inflammation. Previous studies have highlighted the role of NK T cells in oxazolone colitis; however our data suggests that the pathogenesis is dependent on CD4<sup>+</sup> T cells expressing IL-4Rα. It is possible that NK T cells induce the pathology associated with colitis by driving CD4<sup>+</sup> T cells to produce IL-13. However the reverse would also be possible, with CD4<sup>+</sup> T cells driving NK T cells to induce pathology. Therefore, depletion of NK T cells or blocking IL-13 would also result in protection from disease onset.

The induction of oxazolone colitis depends on a variety of immunological processes and a better understanding of the role of IL-4 and IL-13 on individual cells would elucidate disease pathology. A recent paper in our lab (Horsnell *et al.* 2007) has shown the importance of IL-4/IL-13 responsive smooth muscle cells in the expulsion of the nematode *Nippostrongylus brasiliensis*. Mice deficient in the IL-4Rα specifically on smooth muscle cells (SM-MHC<sup>cre</sup>IL-4Rα<sup>-lox</sup>) demonstrate delayed worm expulsion, delayed goblet cell hyperplasia and reduced Th2 cytokine production. From this data it was proposed that smooth muscle IL-4Rα is an important amplifier of Th2 cytokines from the mesenteric lymph node and surrounding tissue. If this reflects a general mechanism one might suggest that IL-4/IL-13 responsive smooth muscle cells also play a role in oxazolone colitis. Furthermore, alternatively activated macrophages (aaMph's) have recently been shown to protect against colitis in a mouse model of Crohn's disease (Hunter *et al.* 2009). The role of alternatively or classically activated macrophages has not been investigated in oxazolone colitis. Essentially, classically activated macrophages are induced by Th1 cytokines (IFN-γ) and are associated with tissue injury. In contrast, aaMph's are activated by Th2 cytokines (IL-4 and IL-13) and are associated with the resolution of inflammation and wound healing (Gordon 2003). Considering that Th2

responses are responsible for the inflammation associated with UC, data obtained by using macrophage/neutrophil-specific IL-4R $\alpha$ -deficient mice (LysM<sup>cre</sup>IL-4R $\alpha$ <sup>-lox</sup>) would be of interest in the oxazolone colitis model. On the one hand, an increase in classical macrophage activation leading to a shift towards increased Th1 responses could protect against Th2-induced colitis. On the other hand, aaMph's are associated with tissue repair and preventing their activation could result in a more severe oxazolone-induced inflammation.

In conclusion, several mouse models of IBD have been described, but few of these represent Th2-mediated colitis. By using the oxazolone-induced model of colitis we investigate the immune mechanisms involved in human UC. Genetically manipulated mice allow us to investigate the role of specific genes by loss of function. IL-4R $\alpha$ -deficient mice are a novel way to determine the function of IL-4 and IL-13 on individual cells in IBD and have not previously been described in the oxazolone model of UC. The results presented here clearly show the important role of IL-4R $\alpha$  signalling in oxazolone colitis. In the past years substantial progress has been made in understanding the immunological mechanisms of IBD, resulting in exciting new therapeutic approaches. Given the heterogeneity of UC and the role of many cell types in disease onset, it is possible that blocking various signalling pathways could equally protect from the induction of colitis. Our data describe cellular mechanisms underlying mouse models of oxazolone colitis and the importance of similar mechanisms in human UC need to be confirmed.

### 3.1 References

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## APPENDIX A

### **Reagents:**

#### **Anaesthetic**

1.2 ml	Anaket-V (100 mg/ml) (Centaur labs, Isando)
0.8 ml	Rompun (2 %) (Bayer, Germany)
8.0 ml	PBS (1X)

#### **Blocking Buffer**

20g	Milk powder (spar instant) (2 %)
Make up to 1 L with 1X PBS	

#### **Coating Buffer**

10 ml	10x PBS
8g	BSA (Merck)
Make to 1L in ddH <sub>2</sub> O and pH 9.5	

#### **Dilution Buffer**

10g	BSA (1 %) (Roche)
0.2g	NaN <sub>3</sub> (0.02 %) (Merck)
Make up to 1L with 1X PBS	

#### **FACS Buffer**

0.1%	BSA (Roche)
0.05%	NaN <sub>3</sub> (Merck)
Made up in 1X PBS	

#### **Iscoe's Modified Dulbecco's Medium (IMDM)**

1 tube	IMDM (Gibco)
750 ml	ddH <sub>2</sub> O
81.7 ml	NaHCO <sub>3</sub> (37g / L)
2 ml	Penicillin/streptomycin (500X)
Adjust the pH to 7.2 – 7.4	
Make up to 1 L with ddH <sub>2</sub> O and filter sterilize	

#### **Myeloperoxidase Assay**

0.5%	HTAB (hexadecyl trimethyl ammonium bromide)
1mM	EDTA [HTAB buffer]
1 tablet	O-dianisidine in 8ml ddH <sub>2</sub> O
0.005%	H <sub>2</sub> O <sub>2</sub>
10%	NaN <sub>3</sub>

**Phosphate Buffered Saline (PBS 10X)**

80g	NaCl (1.37M)
2g	KCl (0.03M)
14.4g	H <sub>2</sub> PO <sub>4</sub> (0.01 M)
2.4g	KH <sub>2</sub> PO <sub>4</sub>
Dissolve in 1 L ddH <sub>2</sub> O	

**Red cell lysis buffer**

5 mM	EDTA	150 mM	NaCl
10 %	glycerol	25 mM	Tris-Cl pH 7.5
0.1 %	SDS	1 %	Triton –X 100
0.5 %	Non idet P-40	0.5 %	Deoxycholate
5 mM	PMSF		

Make to 1L with ddH<sub>2</sub>O

**Substrate Buffer**

0.2g	NaN <sub>3</sub> (0.02 %)
97 ml	di-ethanolamine
0.8g	MgCl <sub>2</sub> .6H <sub>2</sub> O
700 ml	ddH <sub>2</sub> O

Adjust the pH to 9.8 and make up to 1 L with ddH<sub>2</sub>O

**Washing buffer**

20g	KCL	20g	KH <sub>2</sub> PO <sub>4</sub>
144g	NA <sub>2</sub> HPO <sub>4</sub> .H <sub>2</sub> O	800g	NaCl (Merck-BDH)
50 ml	Tween 20 (Sigma)	100 ml	10 % NaN <sub>3</sub> (Merck)

Make up to 5 L with ddH<sub>2</sub>O

**Supplementary Methods:****Disease activity index scoring sheet:**

<b>Appearance</b>		<b>Natural Behaviour</b>	
Normal	0	Normal	0
General lack of grooming	1	Minor changes	1
Coat staring. Ocular and nasal discharges	2	Less mobile and alert, isolated	2
Piloerection, hunched up	3	Vocalization, restlessness, very still	3
<b>Clinical signs</b>		<b>Provoked behaviour</b>	
Normal colour & movement	0	Normal	0
Slight changes in activity	1	Minor depression or exaggerated response	1
Moderated Changes: weight loss, diarrhoea	2	Moderate change in exoected behaviour	2
Severe changes: immobility, lameness	3	Reacts violently or very weak	3

\* If 3 was scored more than once, an extra point was scored for every 3. **Total = 16**

**Lung tissue single cell suspensions**

Individual lungs were cut into small pieces using a scalpel blade and incubated in DMEM (Gibco) containing 50U/ml Collagenase I (Gibco-INvitrogen) and 13µg/ml DNase I (Roche, Germany) for 90 min at 37°C. The tissue was pressed through a 70 µm sieve and red blood cells (RBC) were lysed with lysis buffer for 5 min on ice. The remaining cells were collected by centrifugation (1200 rpm for 5 min at 4°C) and stained with CD23, CD19 or CD3 mAbs for FACS analysis.

**Bicinchoninic Acid Protein Estimation (BCA)**

The BCA protein assay (Pierce, Rockford, USA) was used to determine the protein concentration of all samples. The principle of this method is the reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{1+}$  by protein in an alkaline medium with the colorimetric detection of the cuprous cation ( $\text{Cu}^{1+}$ ) by bicinchoninic acid. The rate of BCA colour formation (purple) is dependent on the incubation temperature, the types of protein present in the sample and the amounts of reactive amino acids in the proteins. The protein standard was prepared by diluting Bovine Serum Albumin (BSA) in the same diluent as the samples (diluted 10 fold down). The BCA working solution was added and after incubation at 37 °C for 30 min the absorbance was measured at 540 nm using a microplate spectrophotometer (Molecular devices, California, USA).

**Preparation of histology sections**

Tissues were dehydrated in an automated processor and embedded in wax as indicated below:

70% alcohol	30min
96% alcohol (2x)	45min
100% alcohol (4x)	45min
Xylol (2x)	60min
Wax (55°C-60°C) (2x)	45min (with vacuum)

The tissues were sectioned at 2µm with a microtome, floated onto glass slides and fixed by incubation at 37°C overnight. The wax was removed from sections by incubation at 60°C for 2-18h and then rehydrated as follows:

Xylol	30min
Xylol (2x)	1 min
100% alcohol	1min
96% alcohol	1min
70% alcohol	1min
Water	1min